

Department of Equine and Small Animal Medicine
Faculty of Veterinary Medicine
University of Helsinki
Finland

CANINE IDIOPATHIC PULMONARY FIBROSIS

*CLINICAL DISEASE, BIOMARKERS
AND HISTOPATHOLOGICAL FEATURES*

HENNA P. LAURILA



ACADEMIC DISSERTATION

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Director of studies	<p>Professor Thomas Spillmann, Dipl.med.vet, Dr.med.vet, Dipl. ECVIM-CA Department of Equine and Small Animal Medicine Faculty of Veterinary Medicine University of Helsinki, Finland</p>
Supervisors	<p>Docent Minna Rajamäki, DVM, PhD Department of Equine and Small Animal Medicine Faculty of Veterinary Medicine University of Helsinki, Finland</p> <p>Professor Cécile Clercx, DVM, PhD, Dipl. ECVIM-CA Department of Clinical Sciences Faculty of Veterinary Medicine University of Liège, Belgium</p>
Reviewers	<p>Professor Brendan C. McKiernan, DVM, Dipl. ACVIM-SAIM Veterinary Clinical Medicine Director, Veterinary Teaching Hospital College of Veterinary Medicine University of Illinois, Urbana, Illinois, USA</p> <p>Professor Anja Kipar, Dr.med.vet.habil., Dipl. ECVP, FRCPath, FTA & FVH (Pathologie) Institute of Veterinary Pathology University of Zürich, Switzerland</p>
Opponent	<p>Professor Eleanor C. Hawkins, DVM, Dipl. ACVIM-SAIM North Carolina State University College of Veterinary Medicine Raleigh, North Carolina, USA</p>

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ABSTRACT

Canine idiopathic pulmonary fibrosis (CIPF) is a chronic interstitial lung disease of unknown origin mainly affecting West Highland white terriers (WHWT). It has no curative treatment. Differentiating CIPF from other chronic respiratory diseases is difficult. A measurable biomarker would be an important addition to diagnostics. CIPF shares many clinical features with human idiopathic pulmonary fibrosis (IPF), but the histopathological resemblance of the canine and human diseases has been unclear.

We described the clinicopathological and diagnostic imaging findings in dogs with CIPF and compared them with those of healthy WHWTs. The most typical clinical signs were cough and exercise intolerance. Fine inspiratory crackles, “Velcro crackles”, were characteristic and an abdominal breathing pattern was often present. Despite being hypoxemic, the dogs were commonly bright and alert. Bronchointerstitial opacity was the most common radiographic finding. In high resolution computed tomography, ground glass opacity was a consistent feature, whereas honeycombing and traction bronchiectasis were less common. Bronchoalveolar lavage fluid (BALF) total cell count was elevated in CIPF and bronchial changes were often detected.

We investigated the serum and BALF concentrations of two potential fibrosis biomarkers, endothelin-1 (ET-1) and procollagen type III amino terminal propeptide (PIIINP) in dogs with CIPF, chronic bronchitis (CB), eosinophilic bronchopneumopathy (EBP) and healthy dogs. Serum ET-1 was higher in dogs with CIPF than in healthy dogs, dogs with EBP or dogs with CB. BALF ET-1 was measurable only in dogs with CIPF. BALF PIIINP was higher in dogs with CIPF than in dogs with CB or healthy dogs, but not different from dogs with EBP. Serum PIIINP was not useful in evaluating respiratory diseases in dogs.

We defined the histopathological lesions and their distribution in WHWTs with CIPF and compared them with those of two human interstitial lung diseases. The diseases chosen for comparison were human IPF, the histopathological pattern of which is known as usual interstitial pneumonia (UIP), and nonspecific interstitial pneumonia (NSIP), which is an important differential diagnosis of human IPF. A diffuse mature interstitial fibrosis of varying severity, resembling human NSIP, was seen in the lungs of all dogs with CIPF. The majority of CIPF dogs also had multifocal areas of accentuated subpleural and peribronchiolar fibrosis with occasional honeycombing and profound alveolar epithelial changes, reminiscent of human UIP. Interstitial fibroblastic foci, characteristic of UIP, were not seen in WHWTs. Severe pulmonary lesions were seen more often in the caudal than in the cranial lung lobes.

In this thesis we provide a detailed description of the clinicopathologic and diagnostic imaging features of CIPF and present quantitative values for arterial blood gases and BALF cytology. Our results indicate that serum ET-1 and BALF PIIINP are elevated in dogs with CIPF and could differentiate CIPF from CB. We conclude that CIPF is histopathologically characterised by two types of interstitial fibrosis and shares features of both human UIP and NSIP.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Heikkilä HP, Lappalainen AK, Day MJ, Clercx C, Rajamäki MM. Clinical, bronchoscopic, histopathologic, diagnostic imaging, and arterial oxygenation findings in West Highland white terriers with idiopathic pulmonary fibrosis. *Journal of Veterinary Internal Medicine* 2011, 25: 433-439.

- II Krafft E, Heikkilä HP, Jespers P, Peeters D, Day MJ, Rajamäki MM, Mc Entee K, Clercx C. Serum and bronchoalveolar lavage fluid endothelin-1 concentrations as diagnostic biomarkers of canine idiopathic pulmonary fibrosis. *Journal of Veterinary Internal Medicine* 2011, 25: 990-996.

- III Heikkilä HP, Krafft E, Jespers P, McEntee K, Rajamäki MM, Clercx C. Procollagen type III amino terminal propeptide concentrations in dogs with idiopathic pulmonary fibrosis compared with chronic bronchitis and eosinophilic bronchopneumopathy. *The Veterinary Journal* 2013, 196: 52-56.

- IV Syrjä P, Heikkilä HP, Lilja-Maula L, Krafft E, Clercx C, Day MJ, Rönty M, Myllärniemi M, Rajamäki MM. The histopathology of idiopathic pulmonary fibrosis in West Highland white terriers shares features of both non-specific interstitial pneumonia and usual interstitial pneumonia in man. *Journal of Comparative Pathology* 2013, 149: 303-313.

The publications are referred to in the text by their roman numerals. The original publications are reprinted with the kind permission of their copyright holders. Some unpublished material is additionally presented.

ABBREVIATIONS

ABE	acid base excess
ALP	alkaline phosphatase
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CB	chronic bronchitis
CIPF	canine idiopathic pulmonary fibrosis
CK	cytokeratin
COPD	chronic obstructive pulmonary disease
CT	computed tomography
DAD	diffuse alveolar damage
EBP	eosinophilic bronchopneumopathy
ET-1	endothelin-1
GGO	ground glass opacity
HE	hematoxylin and eosin
HCO ₃ ⁻	bicarbonate
HMW-CK	high molecular weight cytokeratin
HRCT	high resolution computed tomography
HU	Hounsfield unit
IHC	immunohistochemistry
IIP	idiopathic interstitial pneumonia
ILD	interstitial lung disease
IPF	idiopathic pulmonary fibrosis
IQ	interquartile range
NSIP	nonspecific interstitial pneumonia
P(A-a)O ₂	alveolar-arterial oxygen gradient
PaCO ₂	partial pressure of arterial carbon dioxide
PH	pulmonary hypertension
PaO ₂	partial pressure of arterial oxygen
PCI	pneumocyte type I
PCII	pneumocyte type II
PIIINP	procollagen type III amino terminal propeptide
ROC	receiving operating characteristic
SD	standard deviation
SMA	α-smooth muscle actin
SP	surfactant protein
TCC	total cell count
TGF-β	transforming growth factor β
UIP	usual interstitial pneumonia
WHWT	West Highland white terrier

1 INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a devastating interstitial lung disease with no known cure. It is chronic, inevitably progressive and eventually leads to death. IPF is recognised in humans (Liebow, 1975) and in their animal companions, cats (Cohn et al., 2004; Williams et al., 2004) and dogs (Corcoran et al., 1999a; Lobetti et al., 2001; Norris et al., 2005). The term ‘canine IPF’ (CIPF) is used for this disease in dogs with a view to separating the human and canine diseases.

CIPF affects mainly the West Highland white terrier (WHWT), a small, friendly but self-reliant white terrier breed originating from the Highlands of Scotland. It was also in Scotland that the systemic study of CIPF began. After conference reports of an emerging fibrosing pulmonary disease affecting dogs, Corcoran et al. (1999b) published a case report of a Staffordshire bull terrier with idiopathic pulmonary fibrosis. Shortly after this, the clinical and diagnostic features of a chronic pulmonary disease were described in 29 WHWTs (Corcoran et al., 1999a). In 2001, Lobetti et al. (2001) provided another case series of a schipperke, a bull terrier and three Staffordshire bull terriers affected by chronic idiopathic pulmonary fibrosis, and a year later a case report of another WHWT with the disease was published by Webb and Armstrong (2002). After this, articles about CIPF focused on the histopathological features (Norris et al., 2005), findings in high resolution computed tomography (HRCT) (Johnson et al., 2005), concomitant pulmonary hypertension (PH) (Schober and Baade, 2006), surfactant protein (SP) C (Erikson et al., 2009), detailed clinical features (Corcoran et al., 2011), and outcome and prognostic factors (Lilja-Maula et al., 2014a). The most recent research brings new insight into the pathogenesis of the disease by investigating the transforming growth factor β (TGF- β) signalling pathway (Krafft et al., 2014; Lilja-Maula et al., 2014b; Lilja-Maula et al., 2014c), gene expression profiles (Krafft et al., 2013) and chemokine concentrations (Roels et al., 2015a; Roels et al., 2015b).

CIPF has striking similarities to human IPF. The key feature of both diseases is an abnormal accumulation of collagen in the lung parenchyma for no known reason (Raghu et al., 1985; Norris et al., 2005). In both humans and dogs, diseased individuals tend to be old, suffer from unexplained cough and exercise intolerance, and fine inspiratory crackles, so called “Velcro” crackles, are heard on lung auscultation. Neither humans nor dogs can be cured of the disease (Corcoran et al., 1999a; Raghu et al., 2011). In humans, IPF diagnosis usually signifies a worse prognosis than most cancers (Ross et al., 2010).

Diagnosing CIPF poses a challenge for the veterinarian. CIPF is a diagnosis of exclusion and laborious examinations are needed to reach that diagnosis. Differentiating CIPF from its main differential diagnosis, chronic bronchitis (CB), is especially challenging. An accurate fibrosis biomarker available for clinical use would be of great help to the veterinary practitioner.

Many aspects of CIPF warrant further investigation. One question at the centre of attention is the histopathological picture of CIPF and to what extent it resembles that of human IPF. This is especially important considering the potential role dogs could have as a spontaneous animal model of human IPF. Several animal models of induced pulmonary fibrosis have been developed, mainly in small rodents, to study new therapeutic agents and

to unravel the fibrotic pathways of human IPF, but the progression of the disease and the histopathological changes differ when compared to the spontaneous form (Moeller et al., 2008; Wolters et al., 2014). A model of spontaneous disease is currently lacking.

This thesis is aimed at further defining the clinical features of the disease, evaluating the use of two potential fibrosis biomarkers in CIPF diagnostics, and shedding light on the histopathological aspects of CIPF and how it compares to human disease.

2 REVIEW OF THE LITERATURE

2.1 INTERSTITIAL LUNG DISEASE IN DOGS AND HUMANS

CIPF and IPF belong to a large and heterogeneous group of interstitial lung diseases (ILDs). The term is synonymous with diffuse parenchymal lung diseases. ILDs are lung disorders in which the distal lung parenchyma is disrupted. The lung can be grossly divided into two functional parts, the parenchyma and the nonparenchyma. The parenchyma contains the delicate gas-exchange tissue whereas as the nonparenchyma contains the airways, vessels and coarser connective tissue components (Weibel, 1986). The parenchymal interstitium of the lung is an anatomical space outlined by the alveolar epithelial cell and capillary endothelial cell basement membrane. The lung interstitium contains the extracellular matrix of the lung, collagen components, noncollagenous proteins as well as a few interstitial cells such as tissue macrophages, fibroblasts and myofibroblasts (Cosgrove and Schwarz, 2011) (Fig. 1).

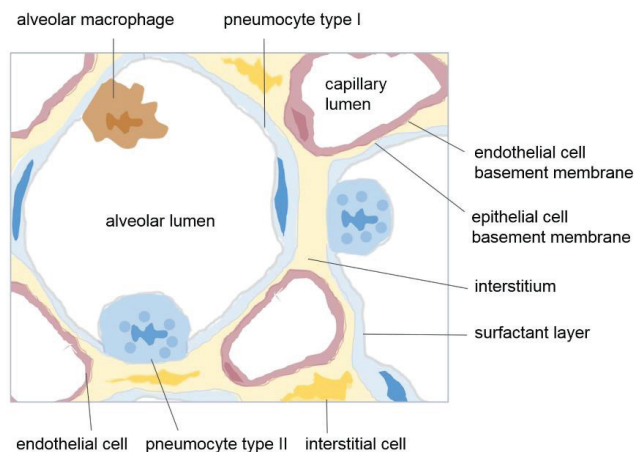


Figure 1 *Schematic illustration of the alveolus and surrounding interstitium.*

More than 200 ILDs are recognised in humans. The word “interstitial” in the term ILD is something of a misnomer, because many ILDs also affect the airways, parenchyma, vasculature and pleura. Although some ILDs are associated with known etiological factors such as inhaled agents, drugs, infections, radiation or systemic diseases such as connective tissue disease, the majority of ILDs have an unknown aetiology (Cushley et al., 1999; Demedts et al., 2001).

Many fewer ILDs are recognised in dogs than in humans (Norris et al., 2002; Reinero and Cohn, 2007). In the veterinary literature, the most often reported ILD in the dog appears

to be eosinophilic bronchopneumopathy (EBP). EBP and CIPF have received much attention in research over the last decade. Only a little is known about the occurrence of other ILDs in dogs and it is mainly only single case reports that have been published. The other reported ILDs in dogs are bronchiolitis obliterans with organising pneumonia, pulmonary alveolar proteinosis, endogenous lipid pneumonia, silicosis and asbestosis (Schuster, 1931; Canfield et al., 1989; Silverstein et al., 2000; Norris et al., 2002). Syrjä et al. (2009) described a familial lung disease in dalmatian dogs clinically resembling acute respiratory distress syndrome but with histopathological changes more similar to human usual interstitial pneumonia (UIP). Because histopathological assessment of lung tissue is often required for ILD diagnosis, EBP being an exception, some ILDs in dogs are likely to go unnoticed (Norris et al., 2002; Reinero and Cohn, 2007).

2.2 CANINE AND HUMAN IDIOPATHIC PULMONARY FIBROSIS

2.2.1 CLASSIFICATION AND DEFINITION

Idiopathic interstitial pneumonias (IIPs) in humans

Hamman and Rich (1935) were the first to describe an unexplained fatal interstitial fibrosis in human patients. An interstitial lung disease more like the one now called IPF began to be increasingly reported only after the 1950s (Cordier and Cottin, 2013). In those early reports, IPF was a broader term which gathered together a spectrum of fibrosing lung disorders. Later, the term ‘IIP’ was introduced and IPF was classified as an IIP disorder. The classification was based on histopathology (Katzenstein and Myers, 1998). The IIPs are a group of non-neoplastic disorders which result from damage to the lung parenchyma by varying patterns of inflammation and fibrosis (American Thoracic Society, European Respiratory Society, 2002). The most recent classification by the American Thoracic Society and European Respiratory Society distinguishes seven major IIPs, two rare IIPs and a set of unclassifiable cases. Major IIPs are grouped into chronic and fibrosing, smoking-related and acute/subacute IIPs. The chronic and fibrosing IIPs are IPF and idiopathic nonspecific interstitial pneumonia (NSIP). IPF is the most common IIP affecting humans, and NSIP is the second most common. The IIP group is not identified as such in veterinary medicine.

Definition of IPF and CIPF

The most recent evidence-based consensus on diagnosis and management of human IPF was published in 2011. The consensus gives joint guidelines from the American Thoracic

Society, European Respiratory Society, Japanese Thoracic Society and Latin American Thoracic Association, and characterises human IPF as follows:

“IPF is defined as a specific form of chronic, progressive, fibrosing interstitial pneumonia of unknown cause, occurring primarily in older adults, limited to the lungs, and associated with the histopathologic and/or radiologic pattern of usual interstitial pneumonia. The definition of IPF requires the exclusion of other forms of interstitial pneumonia including other interstitial pneumonias and ILD associated with environmental exposure, medication, or systemic disease.” (Raghu et al., 2011).

In this definition, the term ‘usual interstitial pneumonia’ is the name used for the histopathological pattern of human IPF.

In veterinary medicine, no consensus on diagnosis or management of CIPF exists. CIPF is also not the only term used to describe the disease. Other names include IPF, chronic IPF, chronic pulmonary fibrosis, canine pulmonary fibrosis, chronic pulmonary disease in WHWTs and ILD in WHWTs (Corcoran et al., 1999a; Lobetti et al., 2001; Webb and Armstrong, 2002; Norris et al., 2005; Schober and Baade, 2006; Erikson et al., 2009; Corcoran et al., 2011). On the whole, the disease refers to a chronic, progressive, fibrosing ILD of unknown cause, occurring mainly in WHWTs. There is no evidence that tissues other than the lung would be affected. The diagnosis is one of exclusion and the histopathological pattern has not been clearly defined.

2.2.2 AETIOLOGY AND PATHOGENESIS

Etiological hypotheses

As the word “idiopathic” implies, the aetiology of CIPF is unknown and the pathophysiology is incompletely understood. In the same way, the aetiology of human IPF remains indefinable. Fortunately, research in the last decade has improved comprehension of the mechanisms, especially behind the human disease (Spagnolo et al., 2015a). An early hypothesis about the aetiology of human IPF suggested that an initial insult to the lung could activate a chronic inflammatory response. The ongoing inflammation would then lead to fibrosis. This idea has been buried: inflammation is not prominent in IPF, it does not seem to be required for the development of a fibrotic response, and anti-inflammatory therapy provides no benefit for human patients (Gross and Hunninghake, 2001; Selman et al., 2001).

The current hypothesis for the aetiology of human IPF focuses on a repetitive insult to distal lung parenchyma followed by an aberrant wound healing process. This is supported by a histopathological feature very characteristic of UIP: both newly formed and old fibroses are seen in the same lung area (Harari and Caminati, 2010). It is not currently known, whether this is also a feature in dogs.

Alveolar epithelial cells and fibroblasts in normal wound healing

Alveoli are lined by two distinct types of epithelial cells, type I alveolar epithelial cells, also called pneumocyte type I (PCI) cells and type II alveolar epithelial cells, also called pneumocyte type II (PCII) cells. PCI cells are thin cells that cover most of the alveolar wall, interface with capillaries and provide a surface for gas exchange. PCI cells are susceptible to injury. PCII cells are larger cuboidal cells located in the corners of the alveoli. PCII cells are multifunctional, in that they serve as progenitor cells for PCI cells, produce surfactant and interact with mesenchymal cells immediately beneath them. PCII cells are more resistant to injury (Selman and Pardo, 2006; Lopez, 2007) (Fig. 1).

In normal wound healing, the restoration of an intact epithelium after an insult is crucial. When PCI cells are injured and sloughed off, the PCII cells undergo hyperplastic proliferation and cover the exposed basement membranes. Some PCII cells then undergo programmed cell death (apoptosis), whereas others differentiate into PCI cells, completing the alveolar repair (Selman and Pardo, 2006; Lopez, 2007).

Fibroblasts are active, spindle-shaped mesenchymal-derived cells. They are very important in any wound healing process. In response to injury, the number of fibroblasts increases at the site of the lesion and some differentiate into myofibroblasts, which are specialised fibroblasts with contractile activity. Fibroblasts and myofibroblasts play a key role in regulating the extracellular matrix turnover by synthesising and degrading its components. During normal healing, the unnecessary fibroblasts and myofibroblasts undergo apoptosis, and normal structure and function is restored (Ackermann, 2007; Ross et al., 2010; King Jr et al., 2011b).

Wound healing gone wrong

Abnormal re-epithelialization, increased epithelial cell death, decreased fibroblast-myofibroblast apoptosis and progressive extracellular matrix accumulation are hallmarks of IPF in humans (Selman et al., 2001). In IPF, the alveolar epithelium has an abnormal morphology and function. There is a loss of PCI cells likely due to injury and increased apoptosis but the PCII cells seem incapable of restoring them. PCII cells appear in increased numbers and are hyperplastic (Selman and Pardo, 2006; King Jr et al., 2011b). The alveolar epithelial cells are abnormally activated and secrete growth factors, such as TGF- β , and cytokines inducing fibroblast proliferation, migration and recruitment of fibroblast progenitor cells. The production of TGF- β also induces the transformation of epithelial cells into fibroblasts in a process called epithelial mesenchymal cell transition, and provokes the differentiation of fibroblasts to myofibroblasts (King Jr et al., 2011b). The myofibroblasts in the IPF lung seem to be resistant to apoptosis. These events lead to an abnormal accumulation of fibroblasts and myofibroblasts, and the exaggerated production of collagen and other extracellular matrix components leading to architectural distortion characteristic of IPF lung.

Although the events discussed above have not been studied in detail in CIPF, there is evidence to support the idea that alveolar epithelium also plays a role in CIPF. Recently,

2 Review of the literature

increased TGF- β signalling activity was detected in the lungs of WHWTs with CIPF. Both Krafft et al. (2014) and Lilja-Maula et al. (2014c) then demonstrated that the majority of TGF- β responsive cells were located in the aberrant alveolar epithelium. This result suggests that abnormally activated alveolar epithelium has a role in the pathogenesis of CIPF, just as it has in human IPF.

Epidemiologic and genetic risk factors

In dogs, no epidemiologic studies evaluating the risk factors of CIPF have been performed. In humans, vigorous research has revealed several potential epidemiologic risk factors for IPF. Increased risk of developing IPF is associated with cigarette smoking, exposure to environmental and occupational agents such as metal and animal dust, gastro-oesophageal reflux, possibly diabetes and chronic viral infections (Baumgartner et al., 1997; Baumgartner et al., 2000; Enomoto et al., 2003; Tang et al., 2003; Raghu et al., 2006; Gribbin et al., 2009). In particular, herpesviruses have been suggested to contribute to the pathogenesis of IPF (Calabrese et al., 2013). A family history of IPF was shown to be the strongest risk factor for human IPF in a recent study (García-Sancho et al., 2011). Despite these findings, no unifying aetiological factor has been discovered (Kottmann et al., 2009).

In dogs, the accumulation of diseased individuals within one breed suggests that CIPF has a strong hereditary background. To date, only one study has examined gene mutations in CIPF. Erikson et al. (2009) analysed surfactant protein (SP) B and SP C from three dogs with CIPF. In one CIPF dog, a Tibetan terrier, the SP C was absent and a mutation in SFTPC exon was detected.

In humans, there is a growing body of evidence suggesting that genetic mutations predispose individuals to the development of IPF, although the vast majority of IPF cases appear to be sporadic. The familial form of IPF accounts for less than 5% of all cases. Mutation in SP C, SP A and in aging-related telomerase genes as well as a potential susceptibility gene called ELMOD2 have been associated with familial IPF (Raghu et al., 2011). Recently, a strong association was confirmed between mucin 5 B gene promoter polymorphism and both familial and sporadic IPF. Genes involved in host defence, cell to cell adhesion and DNA repair have also been shown to contribute to the risk of developing IPF (Seibold et al., 2011; Fingerlin et al. 2013).

Human IPF and probably also CIPF are likely to be the end result of a complex puzzle of endogenous and exogenous risk factors. Both environmental insults and a specific genetic background are required the development of the disease.

2.2.3 CLINICAL DISEASE

CIPF

CIPF affects mainly WHWTs. Dogs of other breeds, mainly terriers, can occasionally be affected. In the veterinary literature, CIPF is also reported in Staffordshire bull terriers, cairn terriers, a Tibetan terrier, a schipperke and a bull terrier (Corcoran et al., 1999a; Corcoran et al., 1999b; Lobetti et al., 2001; Johnson et al., 2005; Erikson et al., 2009).

Dogs tend to be middle-aged or old when they get sick. Corcoran et al. (1999a) reported a mean age of 9 years at the time of diagnosis in a study of 29 WHWTs with CIPF. In other published cases the age has varied from 3 to 16 years. Three of the four reported Staffordshire bull terriers with CIPF were relatively young, at 3-5 years of age (Lobetti et al., 2001).

The incidence and prevalence of CIPF are not known and no estimations have been published. Commonly dogs are already suffering from advanced CIPF when they are presented to the veterinarian for the first time. CIPF is a diagnosis of exclusion and thorough examinations are needed to reach that diagnosis. Histopathologic examination of the lung tissue is the golden standard for diagnosis, however, lung biopsies are seldom taken on living dogs due to their invasiveness, and a histopathologic examination is often only performed after the dog's euthanasia (Heikkilä-Laurila and Rajamäki, 2014).

The described clinical signs are cough, exercise intolerance, breathlessness, dyspnoea, cyanosis, tachypnoea and orthopnoea. The clinical signs develop slowly and the dog's condition deteriorates progressively over months (Corcoran et al., 1999a; Lobetti et al., 2001; Webb and Armstrong, 2002; Johnson et al., 2005; Erikson et al., 2009; Corcoran et al., 2011; Lilja-Maula et al., 2014a).

Lung auscultation reveals diffuse, fine and distinctive inspiratory crackles and sometimes wheezes or rhonchi. No consistent abnormalities have been reported in blood values (Corcoran et al., 1999a). Arterial blood gas analysis can be used to estimate lung function as other lung function tests are not easily available in dogs. Quantitative arterial blood gas values have been published only recently in a limited number of dogs with CIPF (Lilja-Maula et al., 2014a). Elevation of serum C reactive protein is not associated with CIPF (Viitanen et al., 2014).

In thoracic radiographs, a generalised interstitial to bronchointerstitial lung pattern is commonly detected, but the finding is not specific to CIPF. Additionally, mixed alveolar and predominantly bronchial patterns have been reported in association with CIPF. Right-sided cardiac enlargement is present in some dogs indicating cor pulmonale (Corcoran et al., 1999a; Webb and Armstrong, 2002; Johnson et al., 2005). In human IPF, the emphasis of thoracic imaging has shifted from radiography to HRCT which is a much more sensitive imaging technique for detecting changes in the lung parenchyma. Johnson et al. (2005) studied HRCT findings in 10 dogs with CIPF using a classification scheme from human medicine adapted for use in dogs. Dogs with CIPF were found to have the same spectrum of HRCT changes as human patients with IPF. According to Johnson et al. (2005), GGO

appeared in early CIPF, whereas interstitial thickening, traction bronchiectasis and honeycombing became evident at a later stage.

Bronchoscopy and bronchoalveolar lavage (BAL) provide important information about the respiratory tract. Mild to moderate inflammation, mucosal changes such as roughening and thickening, expiratory dynamic airway collapse and tracheal collapse have been observed in dogs with CIPF (Corcoran et al., 1999a; Corcoran et al., 2011). Based on bronchoscopic examination and HRCT findings, Corcoran et al. (2011) suggested that a subset of CIPF cases were more likely to suffer from a bronchial than an interstitial disease, however, this finding requires confirmation: the diseased lung tissue should be examined histopathologically in order to determine whether more prominent airway involvement indicates an alternative diagnosis. There is no report of quantitative BAL fluid (BALF) analysis in dogs with CIPF.

PH is a well known complication in human patients with IPF. Schober and Baade (2006) investigated the use of Doppler echocardiography in predicting PH in WHWTs with CIPF. In their study more than 40% of the WHWTs with CIPF were found to suffer from PH.

Lilja-Maula et al. (2014a) attempted to find prognostic markers which would help predict the course of CIPF. No prognostic markers could be identified. Repeated arterial blood gas measurement and possibly also the distance walked in a six minute walk test could be a means of monitoring disease progression in dogs. Repeated thoracic radiographs do not seem to be very useful, because the radiographic changes may not be related to the change in clinical condition.

Most dogs with CIPF are treated with corticosteroids and bronchodilators, and some also with antibiotics. The concurrent use of azathioprine has been suggested as of additional benefit. There is, however, no proven benefit of the treatment (Corcoran et al., 1999a; Lobetti et al., 2001). No treatment trials have been performed on dogs with CIPF, and the published studies about CIPF in dogs have not been designed to evaluate any treatment effect. There are no reports of using pirfenidone or nintedanib in dogs.

Although the dogs are usually already old when they get sick, CIPF still has a negative impact on their life expectancy. Diseased dogs have an almost five times higher risk of dying than unaffected dogs of the same age. The median survival time varies among individual dogs. A recent study reported a median survival time of 2.7 years from the onset of clinical signs and one year from the diagnosis. Dogs have been suggested to have both a slowly progressive disease course as well as a rapidly progressing one, which is in line with the progression of IPF in humans (Lilja-Maula et al., 2014a).

Human IPF

IPF affects elderly people. Commonly, IPF is diagnosed when patients are in their sixth or seventh decades and diagnosis in patients of less than 50 years of age is rare. Men seem to be more often affected than women (Raghu et al., 2011). The vast majority of IPF cases are sporadic, and familial form accounts for less than 5% of all cases. Clinically and histopathologically these two forms are indistinguishable, except that patients with familial form tend to be younger than those with sporadic disease (Hodgson et al., 2002).

Prevalence and incidence vary, depending on the study population, disease definition and study design. Incidence has been estimated to vary between 5-16 cases per 100,000 persons, and the prevalence between 13-20 cases per 100,000 persons. For unknown reasons, the incidence of IPF is rising (Navaratnam et al., 2011).

The symptoms are slowly progressive exercise induced dyspnoea and cough. Patients are hypoxemic and have restrictive impairment in pulmonary function tests. Fine, bibasilar, end-inspiratory so-called “Velcro” crackles are heard in more than 80% of patients. Polycythaemia is rare despite chronic hypoxemia. Thoracic radiographs show bilateral peripheral reticular opacities (American Thoracic Society, European Respiratory Society, 2000). A UIP pattern in HRCT or in surgical lung biopsy is required for diagnosis. The UIP pattern in HRCT includes patchy areas of reticular changes with honeycombing and traction bronchiectasis located in basal and peripheral lung areas. Ground glass opacity (GGO) should not be extensive (Raghu et al., 2011).

IPF has a poor prognosis. The median survival of patients is only 2.5-3.5 years after diagnosis (King Jr et al., 2011b). The natural history varies: a patient may have a slowly progressive disease, a stable disease with acute periods of worsening, or an accelerated disease (Raghu et al., 2011). Pharmacological treatment options are very limited and for a long time, lung transplantation was the only means to extend the survival of patients (Rosas and Kaminski, 2015). Recommended non-pharmacological therapies include long-term oxygen therapy and pulmonary rehabilitation (Raghu et al., 2011). The standard pharmacological treatment used to be a combination of prednisolone, azathioprine and acetylcysteine but it was found harmful in a recent study (Raghu et al., 2012). Very recently, two novel antifibrotic agents have brought new hope to patients suffering from IPF. These agents, nintedanib and pirfenidone, have been shown to reduce disease progression and are now approved for the treatment of human IPF (King Jr et al., 2014; Richeldi et al., 2014).

2.2.4 HISTOPATHOLOGY

CIPF

The histopathology of CIPF has previously been investigated only in a limited number of dogs. The results have been inconsistent, especially regarding the lesion pattern and the resemblance between pulmonary lesions in dogs and those described in human UIP.

CIPF is characterised by interstitial fibrosis and an accumulation of collagen types I and III in the alveolar septa (Norris et al., 2005). Hyperplastic and abnormal PCII cells and squamous metaplasia have been reported in association with CIPF. Fibroblast foci are a key feature of human UIP, but whether they are a feature of CIPF remains unclear. A fibroblast focus is a subepithelial, convex-appearing aggregate of proliferating fibroblasts and myofibroblasts (Katzenstein et al., 2008). Erikson et al. (2009) described centres of proliferating fibroblasts, but in the study of Norris et al. (2005) no such centres could be detected. The fibrosis pattern in the study of Norris et al. (2005) more resembles the diffuse uniform interstitial fibrosis observed in human NSIP than the patchy and heterogeneous

2 Review of the literature

fibrosis pattern characteristic of human UIP (Katzenstein et al., 2008). Corcoran et al. (1999a) and Erikson et al. (2009) describe a multifocal severe fibrosis, more similar to human UIP.

UIP

Unlike in dogs, in humans the histopathological pattern of IPF has a specific name: UIP. IPF is only used as a clinical term. The word “usual” indicates that the histopathological pattern is the most commonly observed. The word “pneumonia” refers to inflammation rather than infection (Dempsey et al., 2006).

The key feature of human UIP is the heterogeneity of the lesions. Spatial heterogeneity means that areas of less affected or normal lung parenchyma alternate with patchy areas of very severe fibrosis, scarring and honeycomb changes. A honeycomb is a cystic, fibrotic airspace lined by bronchiolar epithelium often filled with mucin. It is a manifestation of scarring and architectural remodelling (Katzenstein et al., 2008). In addition to spatial heterogeneity, the fibrosis is also temporally heterogeneous: Areas of old, collagen rich fibrosis and scattered, small areas of active fibroproliferation coexist in the same lung area. These areas of active fibroproliferation are called fibroblast foci. Fibroblast foci are located in the interstitium, and the fibroblasts and myofibroblasts composing the foci are arranged parallel to the alveolar septa. The presence of fibroblast foci indicates an active, ongoing fibrosis (Katzenstein and Myers, 1998; Katzenstein et al, 2008). Smooth muscle hyperplasia can be seen in areas of fibrosis. The intra-alveolar accumulation of macrophages is a common finding. Inflammation is usually mild to moderate and consists of interstitial infiltrates of lymphocytes, plasma cells and histiocytes associated with PCII hyperplasia. Fibrosis is distributed subpleurally, paraseptally and peripherally (Katzenstein and Myers, 1998; American Thoracic Society, European Respiratory Society, 2000; American Thoracic Society, European Respiratory Society, 2002; Katzenstein et al., 2008).

2.3 NONSPECIFIC INTERSTITIAL PNEUMONIA

NSIP is a chronic and fibrosing IIP described in humans. Such a disease has not been described in dogs to date. As indicated by the name, the findings are nonspecific. NSIP was accepted as a distinct clinical entity only very recently (Travis et al., 2013).

The prevalence and incidence of NSIP are unknown. Affected patients are on average a decade younger than those affected by IPF. The symptoms, cough and dyspnoea, start gradually. A typical HRCT finding is a bilateral GGO with irregular reticular changes and traction bronchiectasis. Unlike in IPF, honeycombing is uncommon and subpleural lung areas are spared (Kligerman et al., 2009; Travis et al., 2013).

Many cases of NSIP are idiopathic, however, the histopathologic pattern of NSIP is also associated with a variety of conditions such as collagen vascular disease, hypersensitivity pneumonitis, drug reactions, dust exposure and familial pulmonary fibrosis (Travis et al., 2013).

NSIP can be divided into two main histologic subtypes, fibrotic and cellular NSIP, the cellular form being much less common than the fibrotic form. The differentiation is based on the degree of inflammation and fibrosis present (Kligerman et al., 2009). The prognosis of NSIP varies and depends on the amount of fibrosis. The cellular form responds well to corticosteroids and the prognosis is excellent. Patients with fibrotic NSIP have a median survival of 6-14 years, which is still clearly better than that of IPF (Kim et al., 2006).

Histopathologically NSIP is characterised by varying amounts of inflammation, and fibrosis with a uniform appearance. Honeycombing is not a feature and fibroblast foci are seen only occasionally, if at all (Katzenstein et al., 2008; Travis et al., 2008).

2.4 CANINE CHRONIC BRONCHITIS

CB is one the most common chronic respiratory diseases affecting dogs (McKiernan, 2000). It is also the main differential diagnosis of CIPF (Corcoran et al., 1999a). CB is characterised by chronic inflammation of the airways, thickening of the bronchial walls and mucus hypersecretion. The key feature is a chronic, inexplicable cough occurring on most days in two consecutive months in the preceding year (Pirie and Wheeldon, 1976).

The aetiology of the disease is poorly understood, but inhaled environmental irritants, tobacco smoke, low-grade infection, ongoing inflammation and genetic defects are suggested to play a role (McKiernan, 2000; Kuehn, 2004).

CB mainly affects middle-aged to older small breed dogs, such as terriers, but bigger dogs can also be affected (Pirie and Wheeldon, 1976; Padrid et al., 1990). In addition to a cough, the affected dogs may also suffer from exercise intolerance, expiratory dyspnoea and even syncope (Kuehn, 2004; Rozanski, 2014).

CB is diagnosed by ruling out other chronic cardiac and respiratory diseases. Thoracic auscultation may reveal bronchovesicular sounds, crackles or wheezes but can also be unremarkable (McKiernan, 2000, Kuehn, 2004). Thoracic radiographs typically show thickening of the bronchial walls but can also be normal (Padrid et al., 1990; Mantis et al., 1998). Computed tomography (CT) findings reported are bronchiectasis, bronchial wall thickening, ground glass opacity and peribronchiolar thickening (Szabo et al., 2015). Bronchoscopic changes include excessive mucus in the airways, hyperemic mucosal membranes and an irregular or polypoid appearance of the mucosa. Dogs may also show a partial collapse of the bronchi. In BALF, neutrophils are commonly increased (Padrid et al., 1990; Rozanski, 2014).

CB can lead to hypoxemia. In a previous study of 18 dogs with CB, 40% of the dogs were reported to be hypoxemic. The mean partial pressure of arterial oxygen (PaO₂) was 84 mmHg and it ranged from 67 to 110 mmHg before treatment was instituted (Padrid et al., 1990).

Similarly to CIPF, CB is an incurable disease, however, in many dogs the disease progression and the clinical signs can be controlled by glucocorticoid therapy which alleviates the airway inflammation. Bronchodilators may be of benefit for some individuals although they may not improve arterial blood oxygen values (Padrid et al., 1990; Rozanski, 2014).

2.5 CANINE EOSINOPHILIC BRONCHOPNEUMOPATHY

EBP is characterised by eosinophilic inflammation of the airways and pulmonary parenchyma. The disease is also referred to as pulmonary eosinophilia and pulmonary infiltration with eosinophils (Corcoran et al., 1991; Clercx et al., 2000; Rajamäki et al., 2002). The cause for these eosinophilic infiltrations remains incompletely understood. Immunologic hypersensitivity to aeroallergens with a T helper 2 dominant immune response has been suggested (Clercx et al., 2002; Peeters et al., 2005). The diagnosis relies on documenting eosinophilic inflammation in BALF or bronchial biopsies, and ruling out known causes of eosinophilia (Clercx and Peeters, 2007).

Unlike CIPF and CB, EBP usually affects young adult dogs. Siberian huskies and Alaskan malamutes seem to be predisposed, but a dog of any breed and size can be affected. Female dogs are affected more often than male dogs (Clercx and Peeters, 2007).

Most dogs cough. Gagging, retching, exercise intolerance, dyspnoea, sneezing and nasal discharge can accompany the cough. Thoracic auscultation often reveals increased lung sounds, wheezes or crackles, but it can also be normal. In thoracic radiographs, moderate to severe bronchointerstitial pattern, peribronchial cuffing, alveolar infiltrates and bronchiectasis can be present (Corcoran et al., 1991; Clercx et al., 2000; Rajamäki et al., 2002). A recent study looked into the CT images of EBP dogs and reported pulmonary parenchymal abnormalities, bronchial wall thickening, plugging of the bronchial lumen, bronchiectasis, pulmonary nodules and lymphadenopathy (Mesquita et al., 2015). In bronchoscopy, an increased amount of yellow to green or blood-tinged mucus, thickened mucosa with an irregular appearance, hyperaemia and sometimes expiratory airway closure can be observed (Clercx and Peeters, 2007). In addition to BALF eosinophilia, approximately 50% of dogs also have eosinophilia of the peripheral blood. Hypoxemia can be present, but appears not to be very common (Rajamäki et al., 2002).

The hallmark of EBP treatment is glucocorticoid therapy. Some dogs are cured, whereas in other dogs relapses may occur after discontinuation of treatment. The general prognosis is good (Clercx et al., 2000; Rajamäki et al., 2002).

2.6 BIOMARKERS OF CIPF

As already mentioned, diagnosing CIPF requires extensive diagnostic work, but differentiating CIPF from CB or other chronic lung diseases can remain a challenge for the veterinarian. It is especially challenging to diagnose CIPF in its early phase when signs and findings are likely to be subtle. A fibrosis biomarker could therefore be helpful in resolving dilemmas related to diagnostics.

A biological marker (biomarker) is any substance or feature that can be objectively measured and quantified from an individual, or from biological fluids, tissues, or from an individual themselves and serves as an indicator of normal biological or pathogenic processes (Atkinson et al., 2001). A good biomarker is sensitive and specific, easily obtained and practical to use (Atkinson et al., 2001; Louhelainen et al., 2008; Ley et al., 2014). An ideal biomarker would also have prognostic value and could help in uncovering

some of the pathomechanisms of the disease. At the moment there are several candidate biomarkers for human IPF, but none has an established role in clinical practice (Ley et al., 2014).

Biomarker research in CIPF

In dogs, different investigational approaches have been used in an attempt to identify potential biomarkers and to gain insight into the pathomechanisms of CIPF. We previously demonstrated by zymography that CIPF dogs had enhanced gelatinolytic activity in BALF. Matrix metalloproteinase -2 and -9 activities were higher than in dogs with CB (Heikkilä et al., 2011). Lilja-Maula et al. (2013) made a comparison of BALF proteomes between dogs with CIPF, CB and healthy dogs. The proteomic changes were similar in CIPF and CB dogs and no CIPF-specific proteins were identified. Activin B, a cytokine of the TGF- β family, was then detected in the BALF of WHWTs with CIPF suffering from acute exacerbation of the disease. Activin B might be a marker of alveolar epithelial damage in CIPF, but further research is needed to confirm this (Lilja-Maula et al., 2014b). Krafft et al. (2013) investigated the pulmonary gene expression from lung samples of dogs with CIPF and healthy control dogs by microarray analysis. A quantitative reverse transcriptase PCR analysis confirmed the change of expression for genes coding chemokine (C-C) ligand (CCL) 2, CCL7, chemokine (C-X-C) ligand 8 (CXCL8), CXCL14, fibroblast activation protein and the palate, lung and nasal associated protein (Krafft et al., 2013). Roels et al. (2015b) showed that serum and BALF CCL2 concentration and BALF CXCL8 concentration were higher in WHWTs with CIPF than in healthy WHWTs. Serum CXCL8 concentration was also higher in healthy WHWTs than in healthy dogs of other breeds (Roels et al., 2015a). Krafft et al. (2014) reported that the serum concentration of TGF- β 1 was elevated in WHWTs with CIPF. The concentration was also higher in healthy WHWTs when compared with healthy dogs of all other investigated breeds except the Scottish terrier.

These results suggest that the chemokines CCL2 and CXCL8 and the profibrotic cytokine TGF- β 1 might be potential biomarkers of CIPF. Because CXCL8 and TGF- β 1 are elevated in both healthy and sick WHWTs but not in dogs of other breeds, these markers could also be related to the breed predisposition of WHWTs to CIPF (Roels et al., 2015a; Roels et al., 2015b).

2.6.1 ENDOTHELIN-1

Endothelin-1 (ET-1) is a vasoactive, proinflammatory and profibrotic peptide. It is a key mediator of fibrosis and involved in the pathogenesis of human IPF. ET-1 is the most abundant of the three isoforms of the endothelin family. The highest ET-1 levels are found in the lung but ET-1 also circulates in the blood (Fagan et al., 2001). ET-1 is synthesised as an inactive form which is then processed to active ET-1. Its secretion is regulated at transcriptional level. ET-1 binds to endothelin specific receptors located in various cells throughout the body (Ross et al., 2010; Swigris and Brown, 2010) (Fig. 2).

2 Review of the literature

In a fibrotic lung, ET-1 orchestrates a wide variety of profibrotic and proinflammatory processes. ET-1, for example, induces the production of TGF- β and other cytokines, has mitogenic effects on vascular and airway smooth muscle cells and fibroblasts, stimulates fibroblast chemotaxis, proliferation and collagen production, decreases collagen degradation, mediates the epithelial to mesenchymal transition and the differentiation of fibroblasts into myofibroblasts (Teder and Noble, 2000; Fagan et al., 2001; Jain et al., 2007; Ross et al., 2010).

Further evidence of the participation of ET-1 in fibrosis process comes from studies in which ET-1 action is blocked by its antagonist. A dual ET antagonist, bosentan, has been shown to attenuate lung collagen accumulation in a bleomycin induced rodent model of pulmonary fibrosis. Bosentan has shown some promise in the treatment of human IPF, but the results of the latest studies have been disappointing (King Jr et al., 2011a; Spagnolo et al., 2015b).

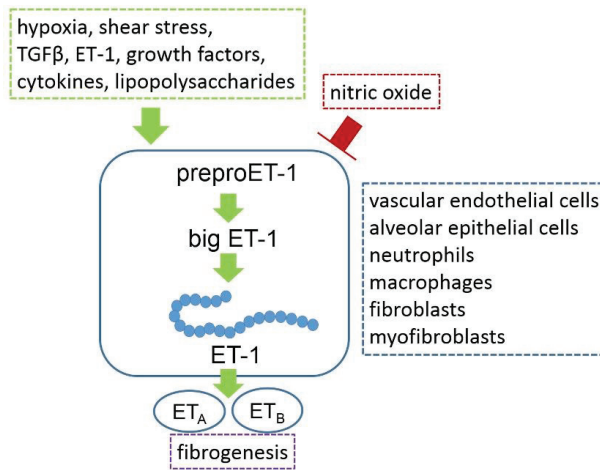


Figure 2 *Simplified illustration of endothelin-1 (ET-1) synthesis. In fibrotic lung, ET-1 is secreted by endothelial and alveolar epithelial cells, neutrophils, macrophages, fibroblasts and myofibroblasts. ET-1 is synthesised as a preprohormone which is processed to big ET-1 and finally to ET-1, a biologically active peptide of 21 amino acids. Synthesis is enhanced by various stimuli such as TGF- β and ET-1 itself, and inhibited by nitric oxide. ET-1 is not stored. Once secreted, its actions are mediated through ET_A and ET_B receptors (Fagan et al., 2001).*

ET-1 as a biomarker in dogs

Commercially available immunoassays for measurement of human ET-1 have been validated for the use in dogs (Schellenberg et al., 2008). Mostly, ET-1 has been investigated as a cardiac biomarker. Blood ET-1 concentration is higher in dogs with cardiac disease or respiratory disease than in healthy dogs, and the concentration rises when cardiac or respiratory disease worsens (Tessier-Vetzel et al., 2006; Piantedosi et al., 2009). Higher

blood ET-1 concentration is detected in dogs with congestive heart failure compared to dogs with cardiac disease but no heart failure (Prošek et al., 2004). Blood ET-1 concentration can be used to differentiate dogs with dyspnoea due to heart failure from those with noncardiogenic dyspnoea (Prošek et al., 2007). An elevated blood ET-1 concentration is also detected in dogs with heartworm disease and in the canine model of ventilator-induced lung injury (Uchide and Saida, 2005; Lai et al., 2010). The concentration of the ET-1 precursor, big ET-1, has also been investigated in dogs, and an increased blood big ET-1 concentration is detected in dogs with chronic kidney disease, neoplastic disorders, cardiac diseases and PH (O'Sullivan et al., 2007; Rossi et al., 2013; Fukumoto et al., 2014). To the author's knowledge, no study has evaluated the use of ET-1 concentration to distinguish between different respiratory diseases in dogs.

ET-1 as a biomarker in humans

Elevated ET-1 concentration in blood and BALF and enhanced tissue expression of ET-1 are well documented in human patients with IPF (Giaid et al., 1993; Sofia et al., 1993; Uguccioni et al., 1995), however, the rise in ET-1 is not specific to IPF and other lung diseases such as asthma, pneumonia, acute respiratory distress syndrome and sarcoidosis have also been associated with increases in blood or BALF ET-1 in humans (Sofia et al., 1993; Letizia et al., 2001; Reichenberger et al., 2001; Gawlik et al., 2006).

Most ET-1 studies have concentrated on the cardiovascular system (Teder and Noble, 2000). ET-1 is known to be a very potent vasoconstrictor and high plasma concentration has been documented in PH and heart failure (Chester and Yacoub, 2014; Gottlieb et al., 2014). ET-1 concentration also increases with age (Komatsumoto and Nara, 1995).

2.6.2 PROCOLLAGEN TYPE III AMINO TERMINAL PROPEPTIDE

Collagens are the most abundant proteins in animals and major elements in the extracellular matrix. They are classified according to their function and structure. Type III collagen is one of the fibrillary collagens which provides tensile strength for connective tissues. During the synthesis of collagen type III, an amino terminal propeptide is proteolytically cleaved from the procollagen molecule to form mature collagen (Fig. 3). This propeptide, the procollagen type III amino terminal propeptide (PIIINP), is then released into extracellular fluid and circulation in proportion to the amount of collagen produced (Prockop et al., 1979; Patino et al., 2002; Kadler et al., 2007).

PIIINP is measurable from various body fluids. Blood PIIINP concentration depends on the rate of its synthesis and can thus be used as a marker for enhanced collagen type III metabolism. In BALF, the levels of PIIINP are likely to represent the local production of collagen in the lung (Lammi et al., 1999).

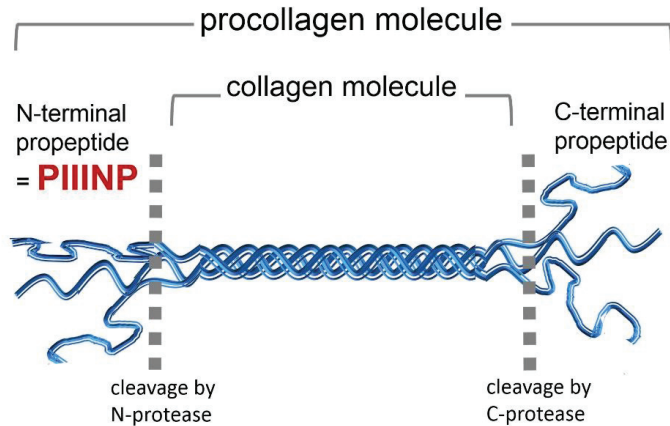


Figure 3 *Schematic illustration of procollagen molecule. After being secreted from the cell, amino (N) terminal and carboxy (C) terminal ends of the procollagen molecule are cleaved by proteases and a mature collagen monomer molecule is formed. The N-terminal propeptide of procollagen type III is called PIIINP.*

PIIINP as a biomarker in dogs

A radioimmunoassay for measuring PIIINP in BALF and serum has been validated in dogs (Schuller et al., 2006). Young, growing dogs have a higher serum and BALF PIIINP concentration than adult dogs. Cardiac diseases and chronic kidney disease do not seem to increase serum PIIINP concentration but collagen type III glomerulopathy is associated with increased values. High BALF PIIINP concentration was reported in a group of dogs with chronic bronchopneumopathy that consisted mainly of dogs with EBP (Schuller et al., 2006; Rortveit et al., 2013). As an abnormal accumulation of collagen types I and III in the pulmonary interstitium is a hallmark of CIPF (Raghu et al., 1985; Norris et al., 2005), PIIINP could be a useful indicator of the disease. PIIINP concentrations have not previously been investigated in dogs with CIPF.

PIIINP as a biomarker in humans

An elevation of PIIINP concentration is detected in the blood and BALF of human patients with IPF (Harrison et al., 1993; Hiwatari et al., 1997; Lammi et al., 1999), however, other respiratory diseases, such as chronic obstructive pulmonary disease (COPD), asthma, sarcoidosis, acute respiratory distress syndrome and infant bronchopulmonary dysplasia have also been associated with increased concentrations or enhanced expression of PIIINP in humans (Lammi et al., 1997; Meduri et al., 1998; Kaarteenaho-Wiik et al., 2004; Kanazawa and Yoshikawa, 2005; Harju et al., 2010).

Blood PIIINP concentration is also affected by a variety of disorders, other than lung diseases. Elevated blood PIIINP concentrations are also associated with growth in healthy young individuals (Lammi et al., 1999).

3 AIMS OF THE THESIS

The objective of this thesis was to describe the clinico- and histopathological findings of CIPF and to determine the usefulness of two fibrosis biomarkers in its diagnosis. The detailed aims were as follows:

1. To describe the clinical signs and findings of physical examination, blood and arterial blood gas analyses, radiography, HRCT, bronchoscopy and BALF cytology in WHWTs with CIPF and compare them with healthy control WHWTs (Study I).
2. To analyse the concentration of ET-1 and PIIINP in the serum and BALF of dogs with CIPF, CB, EBP and healthy dogs, and to determine whether the concentration can be used to differentiate CIPF from other chronic respiratory diseases, namely CB and EBP, in dogs (Studies II and III).
3. To define the histopathological lesions and their distribution in WHWTs with CIPF and to compare them with those of human UIP and NSIP (Study IV).

4 MATERIAL AND METHODS

4.1 SELECTION OF DOGS AND DIAGNOSTIC CRITERIA

4.1.1 DOGS WITH CIPF (STUDIES I, II, III)

Fourteen privately owned WHWTs and one privately owned Scottish terrier with CIPF were prospectively recruited in the years 2007-2009. The dogs were diagnosed either at the Veterinary Teaching Hospital of the University of Helsinki, Finland, or at the Veterinary Teaching Hospital of the University of Liège, Belgium.

The diagnostic evaluation of CIPF dogs consisted of history and physical examination (15/15), haematology (13/15), serum biochemistry (13/15), faecal examination (10/15), arterial blood gas analysis (11/15), thoracic radiography (13/15), echocardiography (13/15), HRCT (7/15), bronchoscopy and BAL (13/15). The CIPF diagnosis was later confirmed by histopathological examination of lung tissue in all dogs. Histopathology was performed either by M.J. Day as described in Study I, or by P. Syrjä, as described in detail in Section 4.6. The signalment, the examinations performed on the individual dogs, and inclusion in the different studies of this dissertation are presented in Table 1.

4.1.2 DOGS WITH CHRONIC BRONCHITIS (STUDIES II, III)

Serum and BALF samples were collected from 22 dogs of various breeds with a diagnosis of CB. Samples were obtained in 2001-2008. Dogs with CB were seen either at the Veterinary Teaching Hospital of the University of Liège, or at the Veterinary Teaching Hospital of the University of Helsinki.

Diagnosis of CB was based on clinical signs (chronic cough) and the findings of thoracic radiography, bronchoscopy and BALF analysis (Kuehn, 2004). Other chronic respiratory and cardiac diseases were excluded. The diagnostic investigation also included haematology (22/22), histopathological examination of bronchial mucosal biopsies (13/22), faecal examination (10/22), arterial blood gas analysis (5/22), echocardiography (5/22) and HRCT (2/22). The signalment, the examinations performed on the individual dogs, and inclusion in the different studies of this dissertation are presented in Table 2.

4.1.3 DOGS WITH EOSINOPHILIC BRONCHOPNEUMOPATHY (STUDIES II, III)

Serum and BALF samples were collected from 15 dogs of various breeds with a diagnosis of EBP. All the dogs were examined at the Veterinary Teaching Hospital of the University of Liège during years 2001-2009.

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The diagnosis of EBP was based on clinical signs, eosinophilia in BALF or bronchial eosinophilic infiltration, and clinical response to glucocorticoids (Clercx and Peeters, 2007). Haematology (15/15), thoracic radiography (14/15), histopathological examination of bronchial mucosal biopsies (14/15) and either faecal examination (9/15) or therapeutic trial with fenbendazole (6/15) were performed. The signalment, the examinations performed on the individual dogs and inclusion in the different studies of this dissertation are presented in Table 3.

4.1.4 HEALTHY CONTROL DOGS (STUDIES I, II, III)

Fourteen clinically healthy privately owned older WHWTs were prospectively recruited at the Veterinary Teaching Hospital of the University of Helsinki in the years 2007-2009.

To confirm their health status, the dogs underwent thorough clinical examinations: history and physical examination (14/14), haematology and serum biochemistry (14/14), faecal examination (14/14), arterial blood gas analysis (12/14), thoracic radiography (14/14), echocardiography (13/14), HRCT (11/14), and bronchoscopy and BAL (11/14).

The serum and BALF samples of 21 healthy laboratory beagles were also collected during 2005-2008. The beagles were owned by the Faculty of Veterinary Medicine of the University of Liège or by the Faculty of Veterinary Medicine of the University of Helsinki.

Physical examination, haematology, serum biochemistry, thoracic radiography and bronchoscopy with BAL were performed for all beagles. Arterial blood gas analysis was obtained from 12 of the 21 beagles. The signalment, the examinations performed on the individual dogs and inclusion in the different studies of this dissertation are presented in Table 4.

Table 1 *CIPF dogs: signalment, clinicopathological examinations and inclusion in the studies of this doctoral dissertation.*

No.	signalment				examinations												inclusion in studies			
	country	breed	age	sex	v.blood	ABG	faeces	THX XR	echo	BAL	HRCT	histo	s ET-1	B ET-1	s PIIINP	B PIIINP	study I	study II	study III	study IV
1	F	whwt	13	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	F	wtwt	12	F	-	-	-	-	-	+	-	+	-	-	-	+	+	-	+	+
3	F	whwt	12	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	F	whwt	10	F	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
5	F	whwt	12	M	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
6	F	whwt	14	f	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-
7	F	whwt	12	M	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
8	F	whwt	11	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	F	whwt	14	M	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
10*	F	whwt	14	M	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+
11	F	whwt	15	M	+	-	-	-	-	+	-	+	-	-	-	+	+	-	+	+
12	F	whwt	11	f	+	+	+	+	+	+	+	+	+	+	+	+	#	+	+	+
13	F	whwt	12	f	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
14	F	whwt	13	F	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+	+
15	F	whwt	8	M	+	+	+	+	+	-	-	+	+	-	+	-	#	+	+	+
16	B	Scottish	15	M	+	-	-	+	-	-	-	+	+	+	+	+	#	+	+	-
17	F	whwt	15	M	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+
18	A	whwt	n	n	n	n	n	n	n	n	n	+	-	-	-	-	-	-	-	+
19	B	whwt	n	n	n	n	n	n	n	n	n	+	-	-	-	-	-	-	-	+
20	N	whwt	n	n	n	n	n	n	n	n	n	+	-	-	-	-	-	-	-	+

In Chapter 5.1 of this dissertation, the clinicopathological analyses of 3 dogs were added to the results of Study I. These dogs are denoted # in the Table 1. Dog No. 10, marked with asterisk (*), corresponds to the dog No.10 in Table 4 (see explanation in Chapter 4.2).

ABG, arterial blood gases; A, Austria; BAL, bronchoalveolar lavage; B, Belgium; B ET-1, BAL fluid endothelin-1; B PIIINP, BAL fluid procollagen type III terminal propeptide; echo, echocardiography; F, Finland or female; f, neutered female; histo, histological examination of lung tissue; HRCT, high resolution computed tomography; M, male; m, neutered male; n, not reported; N, the Netherlands; s ET-1, serum endothelin-1; Scottish, Scottish terrier; s PIIINP serum procollagen type III amino terminal propeptide; THX XR, thoracic radiography; v.blood, venous blood; whwt, West Highland white terrier.

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Table 2 *Dogs with chronic bronchitis: signalment, clinicopathological examinations and inclusion in the studies of this doctoral dissertation.*

No.	signalment				examinations												inclusion in studies			
	country	breed	age	sex	v.blood	ABG	faeces	THX XR	echo	BAL	HRCT	histo BM	s ET-1	B ET-1	s PIINP	B PIINP	study I	study II	study III	study IV
1	F	shetl. sheepd.	10	F	+	+	-	+	+	+	+	-	-	-	+	+	-	-	+	-
2	F	schipperke	14	M	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	-
3	F	labrador	11	M	+	+	-	+	+	+	-	-	-	-	+	+	-	-	+	-
4	F	cairn	9	f	+	+	+	+	-	+	-	-	-	-	+	+	-	-	+	-
5	F	shetl. sheepd.	12	M	+	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-
6	B	ir.setter	11	M	+	-	+	+	-	+	-	-	+	-	-	+	-	+	+	-
7	B	labrador	10	M	+	-	-	+	-	+	-	+	+	-	+	+	-	+	+	-
8	B	mixed	10	f	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-
9	B	dachsh.	9	f	+	-	+	+	-	+	-	+	+	+	+	+	-	+	+	-
10	B	malinois	11	m	+	-	+	+	-	+	-	+	+	-	-	+	-	+	+	-
11	B	brittany	12	M	+	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-
12	B	j. russell	7	f	+	-	-	+	-	+	-	+	+	-	-	+	-	+	+	-
13	B	tervueren	8	m	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-
14	B	old engl. sheepd.	9	M	+	-	-	+	-	+	-	+	-	-	-	+	-	-	+	-
15	B	mixed	4	f	+	-	-	+	-	+	-	+	-	-	-	+	-	-	+	-
16	B	dalmatian	9	F	+	-	-	+	-	+	-	+	-	-	-	+	-	-	+	-
17	B	beagle	6	F	+	-	-	+	-	+	-	+	-	-	-	+	-	-	+	-
18	B	brittany	11	F	+	-	-	+	-	+	-	+	+	+	+	+	-	+	+	-
19	B	dachsh.	12	M	+	-	-	+	-	+	-	+	+	+	+	+	-	+	+	-
20	B	yorkshire	1	F	+	-	-	+	-	+	-	-	+	+	-	-	-	+	-	-
21	B	mixed	2	F	+	-	+	+	-	+	-	-	+	-	-	-	-	+	-	-
22	B	doberman	10	f	+	-	+	+	-	+	-	+	+	+	-	-	-	+	-	-

ABG, arterial blood gases; BAL, bronchoalveolar lavage; B, Belgium; B ET-1, BAL fluid endothelin-1; B PIINP, BAL fluid procollagen type III amino terminal propeptide; cairn terr., cairn terrier; dachsh., dachshund; echo, echocardiography; F, Finland or female; f, neutered female; histo BM, histological examination of bronchial mucosal biopsies; HRCT, high resolution computed tomography; ir.setter, Irish setter; j.russell, Jack Russell terrier; M, male; m, neutered male; old engl. sheepd., old English sheepdog; s ET-1, serum endothelin-1; shetl. sheepd., Shetland sheepdog; s PIINP serum procollagen type III amino terminal propeptide; THX XR, thoracic radiography; v.blood, venous blood; yorkshire, Yorkshire terrier.

Table 3 *Dogs with eosinophilic bronchopneumopathy: signalment, clinicopathological examinations and inclusion in the studies of this doctoral dissertation.*

No.	signalment				examinations												inclusion in studies			
	countrv	breed	age	sex	v. blood	ABG	faeces	THX XR	echo	BAL	HRCT	histo BM	s ET-1	B ET-1	s PIIINP	B PIIINP	study I	study II	study III	study IV
1	B	whippet	5	M	+	-	-	-	-	+	-	-	+	-	-	+	-	+	+	-
2	B	mixed	12	M	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	-
3	B	mixed	7	M	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	-
4	B	mixed	7	F	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-
5	B	fox terr.	8	f	+	-	+	+	-	+	-	+	+	-	-	+	-	+	+	-
6	B	cairn	11	F	+	-	-	+	-	+	-	+	-	-	+	+	-	-	+	-
7	B	husky	9	M	+	-	+	+	-	+	-	+	-	-	+	+	-	-	+	-
8	B	mixed	7	f	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-
9	B	newfound	3	F	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-
10	B	poodle	6	f	+	-	+	+	-	+	-	+	-	-	-	+	-	-	+	-
11	B	husky	1	M	+	-	-	+	-	+	-	+	-	-	+	+	-	-	+	-
12	B	j.russell	5	m	+	-	-	+	-	+	-	+	-	-	+	+	-	-	+	-
13	B	mixed	7	m	+	-	+	+	-	+	-	+	-	-	+	+	-	-	+	-
14	B	labrador	1	M	+	-	-	+	-	+	-	+	+	-	-	-	-	+	-	-
15	B	rottweil.	9	F	+	-	-	+	-	+	-	+	+	-	-	-	-	+	-	-

ABG, arterial blood gases; BAL, bronchoalveolar lavage; B, Belgium; histo BM, B ET-1, BAL fluid endothelin-1; B PIIINP, BAL fluid procollagen type III amino terminal propeptide; cairn, cairn terrier; echo, echocardiography; F, female; f, neutered female; fox terr., fox terrier; histo BM, histological examination of bronchial mucosal biopsies; HRCT, high resolution computed tomography; j.russell, Jack Russell terrier; M, male; m, neutered male; newfound, Newfoundland; rottweil., rottweiler; s ET-1, serum endothelin-1; s PIIINP serum procollagen type III amino terminal propeptide; THX XR, thoracic radiography; v.blood, venous blood.

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Table 4 *Healthy control dogs: signalment, clinicopathological examinations and inclusion in the studies of this doctoral dissertation.*

No.	signalment				examinations												inclusion in studies			
	country	breed	age	sex	v.blood	ABG	faeces	THX XR	echo	BAL	HRCT	histo	s ET-1	B ET-1	s PIIINP	B PIIINP	study I	study II	study III	study IV
1	F	whwt	6	F	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
2	F	whwt	4	m	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
3	F	whwt	9	M	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
4	F	whwt	12	F	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
5	F	whwt	11	f	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+	-
6	F	whwt	8	F	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
7	F	whwt	10	f	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
8	F	whwt	3	f	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
9	F	whwt	9	F	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
10*	F	whwt	14	m	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-
11	F	whwt	11	m	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	-
12	F	whwt	14	F	+	+	+	+	+	-	-	-	+	-	+	-	+	+	+	-
13	F	whwt	10	M	+	+	+	+	+	-	-	-	+	-	+	-	+	+	+	-
14	F	whwt	8	f	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-
15	F	whwt	2	M	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
16	F	whwt	6	F	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+
17	B	beagle	3	M	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
18	B	beagle	6	F	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
19	B	beagle	7	F	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
18	B	beagle	5	M	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
19	B	beagle	8	M	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
20	B	beagle	8	F	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
21	B	beagle	8	F	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
22	B	beagle	7	F	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	-
23	B	beagle	8	F	+	+	-	+	-	+	-	-	+	-	-	-	-	+	-	-
24	F	beagle	10	F	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-
25	F	beagle	10	f	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-
26	F	beagle	10	M	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-
27	F	beagle	6	M	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-
28	F	beagle	6	M	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-
29	F	beagle	12	m	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-
30	F	beagle	12	m	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-
31	F	beagle	10	F	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-
32	F	beagle	10	f	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-
33	F	beagle	7	M	+	+	+	+	-	+	-	-	-	-	-	+	-	-	+	-
34	F	beagle	7	M	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-
35	F	beagle	6	M	+	+	-	+	-	+	-	-	-	-	-	+	-	-	+	-

Dog No. 10, marked with asterisk (), corresponds to the dog No. 10 in the Table 1 (see explanation in Chapter 4.2). ABG, arterial blood gases; BAL, bronchoalveolar lavage; B, Belgium; B ET-1, BAL fluid endothelin-1; B PIIINP, BAL fluid procollagen type III amino terminal propeptide; echo, echocardiography; F, Finland or female; f, neutered female; histo, histological examination of lung tissue; HRCT, high resolution computed tomography; M, male; m, neutered male; THX XR, thoracic radiography; s ET-1, serum endothelin-1; s PIIINP serum procollagen type III amino terminal propeptide; v.blood, venous blood; whwt, West Highland white terrier.*

4.2 SELECTION OF SAMPLES FOR HISTOPATHOLOGICAL STUDY (STUDY IV)

Post-mortem lung samples obtained from 18 WHWTs with CIPF were included in Study IV. Samples from 15 of the 18 WHWTs had been collected at the Department of Veterinary Biosciences of the University of Helsinki. Samples from three additional cases were provided by the Veterinary Teaching Hospital of the University of Liège. These included lung samples from one WHWT from Austria, one from Belgium and one from the Netherlands. The signalment, examinations performed prior to euthanasia and the inclusion of these WHWTs in other studies of this dissertation are shown in Table 1.

One of the WHWTs with CIPF participated in Studies I, II and III as a healthy control dog. At the dates of those studies, this dog had no signs or findings indicating lung disease. The dog was euthanised 31 months later at the age of 16.5 years for reasons other than lung disease (incontinence, poor eyesight and weakness of the pelvic limbs). The diagnosis of mild CIPF was made on necropsy.

Three control cases were included. These were post-mortem lung samples which had been collected during routine necropsy from three WHWTs at the Department of Veterinary Biosciences of the University of Helsinki. The dogs had been euthanised for reasons other than lung disease (intestinal intussusception, joint disease and dementia). All control cases underwent a complete necropsy examination. The signalment, the examinations performed prior to euthanasia and inclusion in other studies of this dissertation are shown in Table 4.

Lung samples from four human patients with clinical, radiological and histopathological findings consistent with UIP, and four human patients with clinical, radiological and histopathological findings consistent with NSIP were included. These were either diagnostic biopsy samples or lung transplantation samples, and they were obtained from the Department of Pathology, University of Helsinki, Finland.

4.3 APPROVAL OF STUDY PROTOCOLS

The protocols of Studies I and III were approved by the Committee of Experimental Animals of Western Finland and the protocol of Study II by the Committee of Animal Experimentation of the University of Liège. The owners of the prospectively recruited WHWTs with CIPF and healthy WHWTs provided written consent for participation.

Ethical permission to obtain informed consent from human patients was received from the Helsinki University Hospital Ethical Board. The permission to use tissue samples from deceased patients was obtained from the National Supervisory Authority of Welfare and Health.

4.4 CLINICOPATHOLOGICAL EXAMINATIONS

The owners of the dogs were questioned about clinical signs and a thorough physical examination was carried out. In WHWTs with CIPF and healthy WHWTs, standardised clinical record forms were used for both purposes.

4.4.1 BIOCHEMICAL, HAEMATOLOGIC AND FAECAL EXAMINATION

Blood was drawn either from the cephalic or jugular vein and placed in plain tubes for serum biochemistry and ethylenediaminetetraacetic acid tubes for haematological analyses.

A 3-day faecal sample was collected by owners and examined for parasites using the Baermann and flotation methods.

4.4.2 ARTERIAL BLOOD GAS ANALYSIS

The samples for arterial blood gas analysis were taken from the femoral or dorsal metatarsal artery. The dogs were not sedated for sample collection and were breathing room air. PaO_2 , partial pressure of arterial carbon dioxide (PaCO_2), alveolar-arterial oxygen gradient (P[A-a]O_2), pH, bicarbonate, and acid base excess were analysed immediately with a blood gas analyser (ABL800 FLEX, Radiometer) at 37°C.

4.4.3 THORACIC RADIOGRAPHY AND ECHOCARDIOGRAPHY

Right and left lateral and ventrodorsal radiographs were obtained from all dogs with CIPF and healthy control WHWTs. From healthy beagles and dogs with CB or EBP, single laterolateral and ventrodorsal radiographs were taken.

Echocardiography was performed by one of the authors of the studies (MMR) (Philips iE33, Philips). All dogs underwent complete echocardiographic examination using standardised left and right lateral views.

4.4.4 HIGH RESOLUTION COMPUTED TOMOGRAPHY

The dogs were pre-medicated with an intramuscular administration of butorphanol (Torbugesic, Fort Dodge Animal Health). Anaesthesia was induced with midazolam

(Midazolam Halemn, Algot Pharma) and propofol (PropoVet, Abbot Logistics). An HRCT study was then performed under isoflurane inhalation anaesthesia (Isoba Vet, Schering-Plough Ltd).

The dogs were laying either in dorsal or ventral recumbency and were ventilated before the HRCT scan. Data was obtained with a helical dual slice scanner (Somatom Emotion Duo, Siemens AG) during the respiratory pause. Slice thickness was 1.0 mm and table movement was 7.5 mm. The images were viewed in Syngo Multi Modality Workplace (Siemens AG) by a one of the authors of study I (AKL), who was blinded to the dog's disease status.

The HRCT findings were categorised according to a classification scheme published previously (Johnson et al., 2005). Each radiographic and HRCT finding was scored as normal, mild, moderate or severe. In HRCT, a subtle lesion which was only present in one lung lobe was assessed as mild. When subtle lesions were present in several lobes or the lesion was distinct it was considered as moderate. A widespread and extensive lesion was evaluated as severe even if present in one lung lobe only. Quantitative CT values of the nondependent parts of the lung lobes were measured and mean CT values were calculated.

4.4.5 BRONCHOSCOPY AND BRONCHOALVEOLAR LAVAGE

Bronchoscopy and BAL were performed with a videoendoscope (Olympus GIF-N180, Olympus Europa GmbH; Fujinon EB-4105, Fujinon Europe) using intravenous propofol anaesthesia (PropoVet, Abbot Logistics; Diprivan, AstraZeneca). Dogs were preoxygenated for 5 minutes before the procedure. All sick and healthy WHWTs were premedicated as for HRCT, but in other dogs various anaesthetic protocols were used. Those included intramuscular or intravenous administration of medetomidine (Domitor, Orion Pharma), dexmedetomidine (Dexdomitor, Orion Pharma), or combinations of acepromazine (Combistress, Phenix SA), midazolam (Dormicum, Roche), buprenorphine (Temgesic, Schering Plough) and methadone (Mephenon, Federa SC).

The presence of tracheal collapse, bronchial mucus, bronchial mucosal irregularity, bronchiectasis and bronchomalacia were reported. At least two different lung lobes were lavaged with sterile, warmed (37°C) saline. The amount of lavage fluid was either 2 mL/kg divided into two aliquots (Finnish dogs) or 60 ml divided into three aliquots (Belgian dogs). The BALF sample was processed as described previously (Clercx et al., 2000; Rajamäki et al., 2001). BALF analysis consisted of total cell count (TCC), differential cell count calculations and a quantitative bacterial culture. The limit of infection was set to bacterial growth of 10^3 CFU/mL (Peeters et al., 2008).

Endoscopic biopsies of bronchial mucosa were obtained from 27 dogs if CB or EBP was suspected. Samples were fixed in 10% neutral buffered formalin, processed routinely, and sections were stained with hematoxylin and eosin (HE).

4.5 ANALYSIS OF FIBROSIS BIOMARKERS (STUDIES II, III)

4.5.1 ENDOTHELIN-1 ANALYSIS

Blood samples were collected in dry tubes, centrifuged immediately and the collected serum was stored at -20°C until analysis. A BALF sample was immediately centrifuged and the supernatant was stored at -80°C within 30 min after BALF sampling until further analysis.

ET-1 analysis was performed with a human sandwich ELISA kit (Endothelin-1 Assay kit, IBL). The method has been validated previously for use in canine serum (Schellenberg et al., 2008). This kit had a sensitivity of 0.23 µg/mL. Before measurement, a pre-step of extraction, using Sep-Pak C-18 column (Waters Corporation, Milford) was required to work on the serum. This step permitted a two-fold concentration of the sample in ET-1. It was also performed on BALF samples to increase the likelihood of ET-1 concentration being above the detection limit.

4.5.2 PIIINP ANALYSIS

Serum and BALF PIIINP concentrations were measured using a commercially available radioimmunoassay (¹²⁵I-PIIINP radioimmunoassay, Orion Diagnostics) which was based on purified human PIIINP. The method has previously been validated for use on canine serum and BALF (Schuller et al., 2006). All measurements were taken in duplicate. The detection limit of the assay was 0.03 µg/L. Values below this were given an artificial value of 0.02 µg/L.

4.6 HISTOPATHOLOGICAL ANALYSIS (STUDY IV)

4.6.1 SAMPLING LOCATION AND PREPARATION OF SAMPLES

The samples chosen for Study IV were collected from the cranial and caudal lung lobes of nine WHWTs with CIPF and from the caudal lobes only in three other cases. For the remaining six dogs with CIPF and for the three control dogs, the location of samples collected was not specified. The samples had been fixed in 10% neutral buffered formalin, embedded in paraffin wax and sectioned (4 µm) for routine HE staining.

Five different lung sections were reviewed from each of the 14 WHWTs with CIPF, and from the controls. Only one section of lung was available for review from four dogs with CIPF.

Based on the histopathological findings in the HE stained sections, one slide with lesions most morphologically compatible with human UIP was selected from each of the 18 cases and stained with Masson's trichrome stain for collagen. Further serial sections of the slide

were subjected to immunohistochemistry (IHC) with antibodies specific for α -smooth muscle actin (SMA) (Clon 1A4 Dako) and desmin (Clone D33 Dako) in order to visualise myofibroblasts and smooth muscle hyperplasia, and cytokeratin (CK) (AE1/AE3 DakoCytomation) and human high molecular weight cytokeratin (HMW-CK) (34 β E12, DakoCytomation) to investigate the epithelial changes.

The human samples were included in the staining and immunohistochemical protocols described above.

4.6.2 HISTOPATHOLOGICAL EXAMINATION

All canine samples were examined for the severity and distribution pattern of interstitial fibrosis, the presence of honeycombing, the temporal appearance of the fibrosis, smooth muscle hyperplasia, PCII hyperplasia and atypia, interstitial inflammation, other inflammatory changes, such as bronchitis and pleuritis, and alveolar luminal changes, including histiocytosis, proteinosis and diffuse alveolar damage (DAD).

The severity of the interstitial fibrosis was graded subjectively as mild, if the approximated thickness of alveolar septae did not exceed that of controls, but the alveolar capillaries in the septae were obscured or partially replaced by interstitial collagen. The fibrosis was graded as moderate if the alveolar septae were up to twice the thickness of those in mild lesions and was graded as severe if exceeding this.

Honeycombing was noted as cystic, fibrotic airspaces, lined by low columnar epithelium expressing HMW-CK, which in the normal lung is expressed only by basal bronchiolar cells renewing bronchial and bronchiolar epithelium.

Dense collagen deposition, staining deep blue with Masson's trichrome stain, with low cellularity showing slender spindle-shaped nuclei consistent with fibrocytes, was interpreted as mature fibrosis. The fibrosis was interpreted as immature if the matrix was light blue or myxoid with Masson's trichrome stain, and the cellularity was increased, consisting of ovoid vesicular nuclei interpreted as fibroblasts.

Myofibroblasts were identified among the fibroblasts based on positive SMA labelling and negative desmin labelling in serial sections.

Cuboidal CK positive cells lining the alveolar luminal surface were interpreted as PCII hyperplasia and spindle shaped, broad based, occasionally multinucleated, CK positive cells in the same location were noted as PCII atypia.

In the nine dogs where all lobes were sampled, the presence or absence of honeycombing and severe fibrosis was noted in each lobe, in order to describe the lobar distribution of the most severe lesions.

4.6.3 COMPARISON WITH HUMAN DISEASE

The histopathological findings in WHWTs with CIPF were compared with the key features of human UIP and NSIP (American Thoracic Society, European Respiratory Society, 2002; Katzenstein et al., 2008; Raghu et al., 2011). Lung samples from human patients with UIP

or NSIP were used to provide the depicted comparison of the histopathological findings in WHWTs with CIPF and human disease.

4.7 STATISTICAL ANALYSIS

Analyses were performed using commercial statistical programmes (PASW Statistics 18.0 for Windows, SPSS Inc.; Statistical Analysis Systems Institute, Cary). Normality was evaluated via the Shapiro-Wilk test. Data was presented as mean and standard deviation (SD) or median and interquartile (IQ) range. For pairwise comparisons, either unpaired Student's t-test (data with Gaussian distribution) or Mann Whitney U-tests (non-normally distributed data) were used. Spearman Rank Correlation was used for all correlations. All comparisons were performed 2-tailed.

In Study II, the influences of age and disease group (CIPF, CB, EBP, healthy beagles, healthy WHWTs) on ET-1 concentrations were analysed by 2-way ANOVA in all groups of dogs together. If the effect of age, group, or both was statistically significant, it was studied further by Mann-Whitney U-test. In Study III, the disease groups were compared with either ANOVA or Kruskal-Wallis one-way ANOVA on ranks. In the latter case, the Mann-Whitney U-test was used for pairwise comparisons.

Receiving operating characteristic (ROC) curves were created to analyse the ability of serum ET-1 concentration and BALF PIIINP concentration to differentiate CIPF from CB, and serum ET-1 concentration to differentiate CIPF from CB and EBP.

The differences were considered statistically significant if $P < 0.05$.

5 RESULTS

5.1 CLINICOPATHOLOGICAL FINDINGS IN DOGS WITH CIPF COMPARED WITH HEALTHY CONTROL WHWTs

The results of 14 WHWTs and one Scottish terrier with CIPF are presented and compared with 14 healthy control WHWTs.

5.1.1 SIGNALMENT, CLINICAL SIGNS AND PHYSICAL EXAMINATION

A summary of the signalment of the dogs with CIPF and healthy control WHWTs is given in Table 5. Details of the individual dogs are shown in Table 1 (CIPF) and Table 4 (healthy WHWTs).

At presentation, ten dogs with CIPF were receiving medications (e.g. prednisolone, theophylline, furosemide, benazepril, pimobendan, trilostane, or thyroxin). One CIPF dog had hyperadrenocorticism, and another had hypothyroidism both of which were controlled with medication. One had a urinary tract infection, and another had pyelonephritis. One control dog was receiving prednisolone and cyclosporin for dermatologic problems.

The most common clinical sign was the combination of cough and exercise intolerance described in 8/15 CIPF dogs. Exercise intolerance as the sole problem was reported in 3/15 and cough in 2/15 dogs. One dog had experienced only gagging and another panting. The mean duration of signs was 14 months (range 2-29 months).

The general condition of 13/15 dogs was good, and the dogs were bright and alert. The remaining two dogs were both dyspnoeic and tachypnoeic, and one of those was also cyanotic. Abdominal breathing was reported in 10/15 dogs with CIPF. Diffuse inspiratory pulmonary crackles described as “Velcro crackles” were detected bilaterally in 12/15 dogs. Two dogs were normal on auscultation and one had ronchi. In three dogs the Velcro crackles were also audible without a stethoscope. None of the dogs had wheezes on auscultation.

The control dogs did not have any signs or findings indicating lung disease.

5 Results

Table 5 *Summary of the signalment.*

	CIPF n = 15	CB n = 22	EBP n = 15	healthy WHWT n = 14	healthy beagle n = 21
Sex					
♂/ neutered ♂	8 / 0	9 / 1	6 / 2	2 / 3	9 / 2
♀/ neutered ♀	4 / 3	6 / 6	4 / 3	5 / 4	8 / 2
Age (years) mean ± SD (range)	12 ± 2 (8-15)	9 ± 3 (1-15)	6 ± 3 (1-12)	9 ± 3 (3-14)	8 ± 2 (3-12)
Weight (kg) mean ± SD (range)	10 ± 2 (8-13)	22 ± 14 (4-49)	21 ± 11 (8-44)	9 ± 1 (8-12)	15 ± 2 (9-19)

CB, chronic bronchitis; CIPF, canine idiopathic pulmonary fibrosis; EBP, eosinophilic bronchopneumopathy; WHWT, West Highland white terrier.

5.1.2 BIOCHEMICAL, HAEMATOLOGICAL AND FAECAL EXAMINATIONS

Biochemical and haematological samples were obtained from 13/15 CIPF dogs and 14/14 controls. The following dogs were then excluded from analyses: four CIPF dogs and one control dog receiving prednisolone, one CIPF dog with hyperadrenocorticism and one CIPF dog with pyelonephritis. The analyses were therefore performed on 7 dogs with CIPF and 13 control dogs.

Only the serum concentration of alkaline phosphatase (ALP) and platelet count were not within reference ranges. The ALP concentration was increased in 6 of the remaining 7 dogs with CIPF and in 5 of the remaining 13 control WHWTs. Thrombocytosis was seen in 6/7 dogs with CIPF and in 12/13 controls. The mean ALP concentration was 454 U/L (range 190-827 U/L) in CIPF and 282 U/L (range 54-654 U/L) in controls (reference range 33-215 U/L). The mean thrombocyte count was 534,000/μL (range 370,000-720,000/μL) in CIPF and 556,000/μL (range 356,000-880,000/μL) in controls (reference range 102,000-395,000/μL). The mean haematocrit was 52% (range 33-61%) in CIPF and 51% (range 38-59%) in controls (reference range, 38-57%). The ALP concentration, thrombocyte count, and haematocrit did not differ between the dogs with CIPF and healthy WHWTs. All faecal samples were negative for parasites.

5.1.3 ARTERIAL BLOOD GAS ANALYSIS

Arterial blood gases were obtained from 11/15 dogs with CIPF and 12/14 control dogs. The results are given in Table 6.

Table 6 Arterial blood gas values in WHWTs with CIPF (n=11) and healthy control WHWTs (n=12), given as mean \pm SD and range.

	Dogs with CIPF	Control WHWTs
PaO ₂ (mmHg)*	60.8 \pm 15.4 (33.5-87.4)	99.1 \pm 7.8 (89.6-113.0)
P(A-a)O ₂ (mmHg)*	55.3 \pm 16.5 (28.0-84.7)	17.5 \pm 4.9 (10.7-26.8)
PaCO ₂ (mmHg)	29.2 \pm 3.9 (25.0-35.7)	28.7 \pm 3.8 (20.5-34.6)
pH	7.454 \pm 0.036 (7.407-7.529)	7.467 \pm 0.053 (7.407-7.569)
HCO ₃ ⁻ (mmol/L)	21.0 \pm 2.5 (17.4-25.9)	20.4 \pm 1.9 (17.6-23.0)
ABE (mmol/L)	-1.7 \pm 2.5 (-6.0-3.1)	-1.5 \pm 2.1 (-5.0-1.3)

ABE, acid base excess; CIPF, canine idiopathic pulmonary fibrosis; HCO₃⁻, bicarbonate; P(A-a)O₂, alveolar-arterial oxygen gradient; PaCO₂, partial pressure of arterial carbon dioxide; PaO₂, partial pressure of arterial oxygen; WHWT, West Highland white terrier.

* Statistically significant difference, $P < 0.001$

5.1.4 THORACIC RADIOGRAPHY AND ECHOCARDIOGRAPHY

Thoracic radiographs were taken from 13/15 dogs with CIPF and all control dogs. The most common radiographic finding was bronchointerstitial pattern seen in 8/13 CIPF dogs (moderate in 3/13, severe in 5/13) (Fig. 4). Three of thirteen CIPF dogs had patchy alveolar opacities with indistinct margins in one or two lung lobes. The most common pattern in controls was mild bronchial or bronchointerstitial pattern, which was considered normal. Mean vertebral heart score was 10.2 in both groups (range 9.5-11.1 in CIPF and 9.3-10.8 in controls).

Echocardiography was performed on 10/13 dogs with CIPF and all control dogs. It disclosed right-sided cardiac enlargement in 7/10 CIPF dogs. Tricuspid valve dysplasia and pulmonary stenosis were eliminated in all dogs. Left-sided heart disease was also not detected.

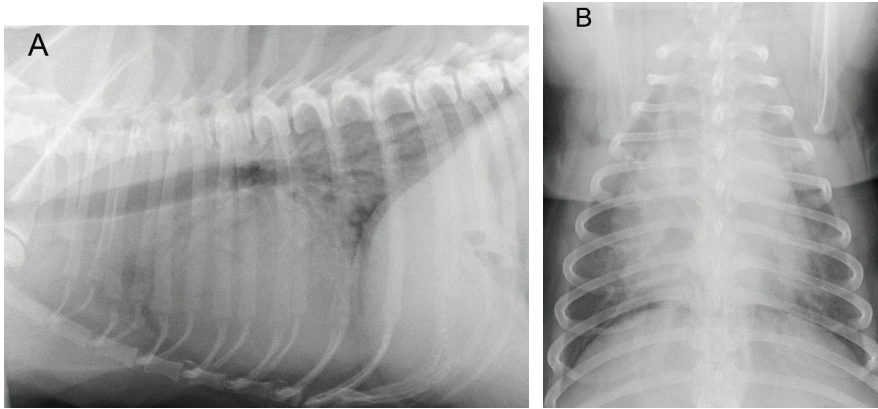


Figure 4 *Right lateral (A) and dorsoventral (B) radiographs obtained from a 12 year old male West Highland white terrier with canine idiopathic pulmonary fibrosis (PaO_2 82 mmHg). The radiographs show a moderate (B) to severe (A) generalised bronchointerstitial lung pattern and a large cardiac shadow.*

5.1.5 HIGH RESOLUTION COMPUTED TOMOGRAPHY

HRCT was performed on 7/15 dogs with CIPF and 11/14 control dogs. On HRCT, several lesions were detected in each dog with CIPF. In the majority of the dogs, the predilection site for the lesions was the dorsocaudal lung lobes. GGO was seen in all dogs (severe in 4/7, moderate in 3/7). Parenchymal bands were visible in 5/7 dogs (mild in 1/7, moderate in 3/7, severe in 1/7), subpleural lines in 2/7, moderate subpleural interstitial thickening in 1/7, moderate peribronchovascular interstitial thickening in 3/7, consolidation in 5/7, traction bronchiectasis in 5/7, and honeycombing in 2/7 dogs. Traction bronchiectasis and honeycombing occurred simultaneously in 2/7 dogs.

In 4/11 controls, no changes were recorded. Eight of 11 controls had only one or two focal lesions in the dependent part of the lung. Consolidation, traction bronchiectasis, subpleural lines, subpleural interstitial thickening, and honeycombing were not seen in any of the controls (Fig. 5).

The CT values (given as Hounsfield units, HU) were significantly higher in CIPF (mean $-731 \pm \text{SD } 51$ HU, range -695 to -786 HU) compared with the controls (-821 ± 36 HU, -761 to -862 HU, respectively; $P < 0.001$). No correlation with PaO_2 was detected.

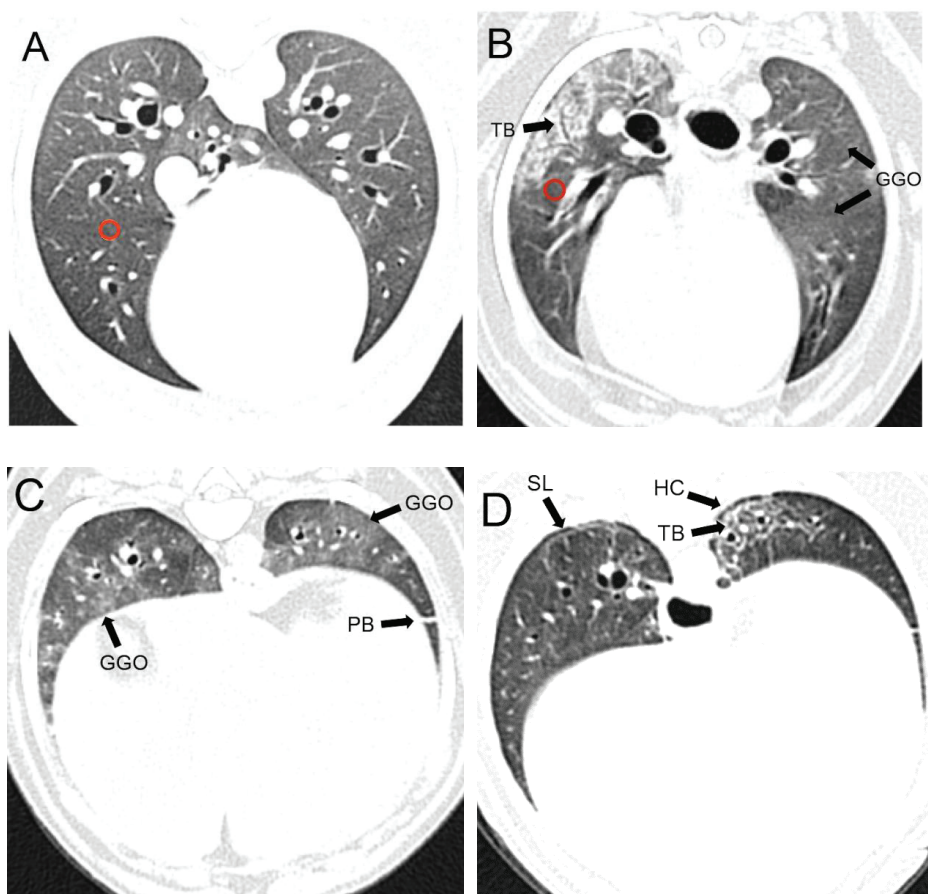


Figure 5 (A) Normal lung. High resolution computed tomography (HRCT) image of the lung of a healthy, 8 year old West Highland white terrier (WHWT). The red circle represents one of the investigated regions of interest (ROI). CT-value was here -848 Hounsfield units (HU). (B) Canine idiopathic pulmonary fibrosis (CIPF). The HRCT image demonstrates areas of ground glass opacity (GGO) and traction bronchiectasis (TB). CT-value of the ROI shown here was -707 HU. The scan was obtained from a 10 year old WHWT with severe hypoxemia (partial pressure of arterial oxygen [PaO_2] 50.6 mmHg). Both dogs (A, B) were scanned on dorsal recumbency. (C) CIPF. The HRCT image shows a parenchymal band (PB) and areas of GGO. The scan was obtained from an 11 year old WHWT (PaO_2 61.3 mmHg). (D) CIPF. The HRCT image illustrates a subpleural line (SL) and an area of honeycombing (HC) and TB. This dog was a 14 year old WHWT that was not hypoxemic (PaO_2 87.4 mmHg). These dogs (C, D) were scanned on ventral recumbency.

5.1.6 BRONCHOSCOPY AND BRONCHOALVEOLAR LAVAGE

Bronchoscopy and BAL were performed on 13/15 dogs with CIPF and 11/14 control dogs. Bronchoscopic results were available only from 9 dogs with CIPF and all control dogs. The

5 Results

findings in dogs with CIPF were bronchial mucosal irregularity (9/9), tracheal collapse (6/9) (grade I in 3/9, grade III in 3/9), mild to moderate amount of bronchial mucus (3/9), mild bronchiectasis (3/9 dogs), and bronchomalacia (5/9) (Fig. 6). Three of 11 controls had grade I tracheal collapse. Mild mucosal irregularity was seen in 7/11 controls.

BALF results were available from 12 dogs with CIPF and 12/14 control dogs. Analyses of BALF are shown in Table 7. BALF TCC, macrophages, neutrophils, mast cells, and mast cell percentage correlated negatively with PaO₂ ($\rho = -0.649$, $P = 0.002$; $\rho = -0.593$, $P = 0.007$; $\rho = -0.596$, $P = 0.006$; $\rho = -0.539$, $P = 0.017$; $\rho = -0.492$, $P = 0.032$, respectively). Lymphocyte percentage had a positive correlation with PaO₂ ($\rho = 0.642$, $P = 0.003$). No bacterial growth was detected.

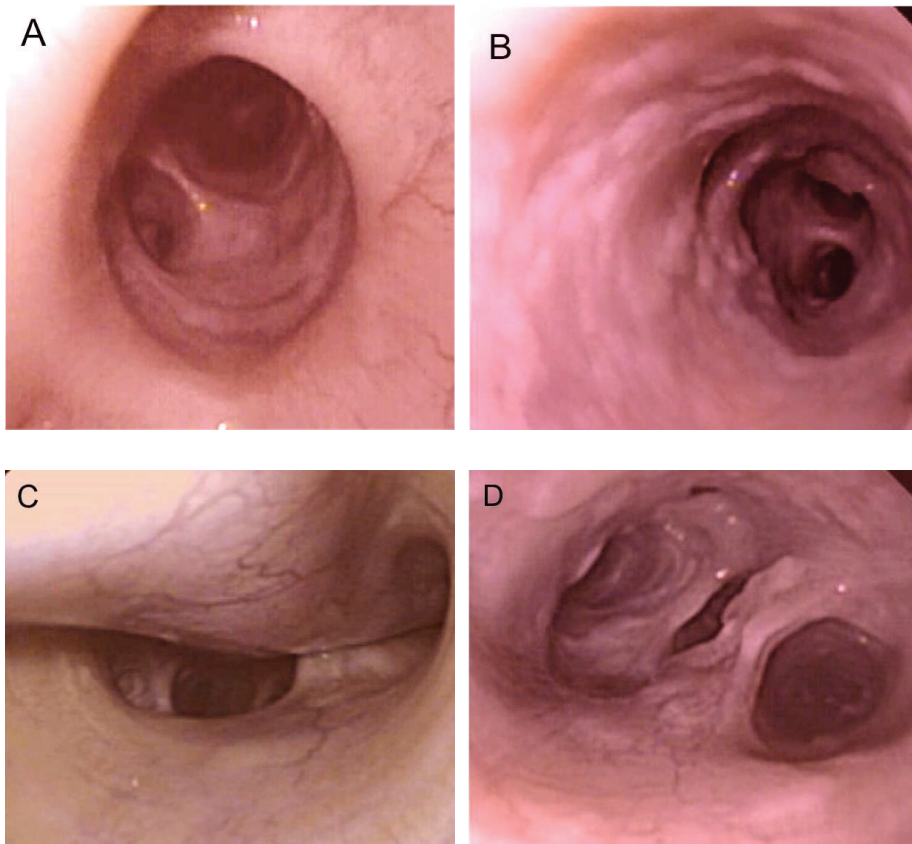


Figure 6 (A) Bronchoscopic view of a normal bronchus. The image was obtained from a 4 year old healthy West Highland white terrier (WHWT). (B) Bronchial mucosal irregularity of moderate severity detected in a 12 year old WHWT with canine idiopathic pulmonary fibrosis (CIPF). (C) Grade I tracheal collapse viewed at the level of carina in a 12 year old WHWT with CIPF. (D) Severe bronchial mucosal irregularity with mild bronchiectasis. The image was taken from a 13 year old WHWT with CIPF.

Table 7 Cytological findings in BALF of WHWTs with CIPF ($n = 12$) and healthy control WHWTs ($n = 11$), given as median, IQ, and range.

	CIPF dogs	Control WHWTs
Recovered volume (%)	55, IQ 49-69 (35-65)	56, IQ 49-69 (40-76)
Total cell count (cells/μL)**	783, IQ 455-1075 (280-3115)	350, IQ 280-380 (265-420)
Macrophages (%)	84, IQ 66-87 (64-93)	78, IQ 76-82 (69-89)
cells/μL**	669, IQ 324-957 (178-2500)	269, IQ 225-289 (194-375)
Lymphocytes (%)*	6.4, IQ 4.8-11 (1.7-30)	16, IQ 14-19 (9.2-21)
cells/μL	51, IQ 29-79 (14-335)	56, IQ 42-63 (39-79)
Neutrophils (%)	4.4, IQ 3.5-21 (3.0-30)	4.5, IQ 3.1-4.7 (0.9-6.2)
cells/μL**	41, IQ 25-120 (10-753)	14, IQ 11-17 (2.8-18)
Eosinophils (%)*	0.0, IQ 0.0-0.0 (0.0-2.0)	0.4, IQ 0.0-0.6 (0.0-2.0)
cells/μL	0.0, IQ 0.0-1.8 (0.0-14)	1.5, IQ 0.0-1.8 (0.0-5.6)
Mast cells (%)*	0.5, IQ 0.4-0.9 (0.0-2.5)	0.3, IQ 0.0-0.4 (0.0-0.9)
cells/μL**	3.9, IQ 1.4-11 (0.0-64)	1.1, IQ 0.0-1.6 (0.0-3.2)
Epithelial cells (%)	0.0, IQ 0.0-1.2 (0.0-9.7)	0.2, IQ 0.0-0.4 (0.0-1.8)
cells/μL	0.0, IQ 0.0-6.3 (0.0-78)	0.9, IQ 0.0-1.4 (0.0-6.3)
Plasma cells (%)	0.0, IQ 0.0-0.0 (0.0-0.6)	0.0, IQ 0.0-0.0 (0.0-0.6)
cells/μL	0.0, IQ 0.0-0.0 (0.0-3.3)	0.0, IQ 0.0-0.0 (0.0-2.1)

BALF, bronchoalveolar lavage fluid; CIPF, canine idiopathic pulmonary fibrosis; IQ, interquartile range; WHWT, West Highland white terrier.

* Statistically significant difference, $P < 0.05$

** Statistically significant difference, $P < 0.01$

5.2 CLINICOPATHOLOGICAL FINDINGS IN OTHER DOGS

5.2.1 SIGNALMENT

A summary of the signalment of the dogs is given in Table 5. Details of the individual dogs are shown in Table 2 (CB), Table 3 (EBP) and Table 4 (healthy beagles).

5.2.2 DOGS WITH CHRONIC BRONCHITIS

All 22 dogs with CB suffered from coughing, the mean duration of which was 11 months (range 2-60 months). Two dogs also had intermittent dyspnoea. All dogs had changes in thoracic radiographs, the most frequent being moderate to severe bronchointerstitial opacity. Alterations were evident in all dogs on bronchoscopy, including an increased amount of mucus (14/22), hyperaemia (11/22), mucosal irregularity (10/22), bronchomalacia (6/22), mucosal thickening (3/22), tracheal collapse (3/22) and focal bronchiectasis (1/22). There was no significant bacterial growth from BALF. The median value for BALF TCC was 500 cells/ μ L (IQ 403-672 cells/ μ L, range 93-12000 cells/ μ L) and the median percentage of neutrophils was 33% (IQ 3-47%, range 1-90%). In the histopathology of the bronchial mucosa, no eosinophilic infiltration was observed. No parasites were detected on faecal examination.

5.2.3 DOGS WITH EOSINOPHILIC BRONCHOPNEUMOPATHY

Dogs with EBP all had coughing as their main clinical sign. Four dogs were also exercise intolerant and three had dyspnoea. The mean duration of clinical signs was 11 months (range 1 - 26 months). Blood eosinophilia (eosinophil count > 750 cells/ μ L was evident in 5/15 dogs. On thoracic radiography, bronchial opacity was detected in all but one dog. This dog had nodular opacities as the sole finding. Interstitial opacities were visible in 8/15 dogs. One dog also had focal alveolar infiltrates.

Bronchoscopic changes were present in all dogs including an increased amount of mucus (12/15), mucosal irregularity (8/15), congestion (4/15), mucosal thickening (3/15) and bronchospasm (1/15). The median TCC in BALF was 1580 cells/ μ L (IQ 925- 4175 cells/ μ L, range 400-12900 cells/ μ L) and the median percentage of eosinophils was 65% (IQ 30-73%, range 20-84%). In one dog the eosinophilic percentage was relatively low, 8%, but infiltration of eosinophils was detected in bronchial biopsy. There was no significant bacterial growth in BALF and no parasites were detected in faecal examination.

5.2.4 HEALTHY BEAGLES

The healthy beagles had no clinical signs, and no abnormalities indicating disease were detected in any of the ancillary examinations.

5.3 ENDOTHELIN-1 ANALYSIS (STUDY II)

5.3.1 SERUM ENDOTHELIN-1 CONCENTRATION

Serum ET-1 concentration from 12 dogs with CIPF was analysed, 13 healthy control WHWTs, 9 healthy control beagles, 10 dogs with CB and 6 dogs with EBP. The results and the comparison of serum ET-1 concentration between dogs with CIPF and dogs with CB, EBP, healthy beagles and healthy WHWTs are shown in Fig. 7. There was no difference between healthy WHWTs and beagles ($P = 0.063$). Serum ET-1 concentration was significantly lower in EBP dogs than in healthy WHWTs ($P = 0.001$) and healthy beagles ($P = 0.011$).

In dogs with CIPF, no correlation was found between serum ET-1 concentration and the duration of clinical signs, PaO_2 , P(A-a)O_2 , or BALF TCC.

The results of ROC curve analysis of serum ET-1 concentration for distinguishing CIPF from CB is illustrated in Fig. 8.

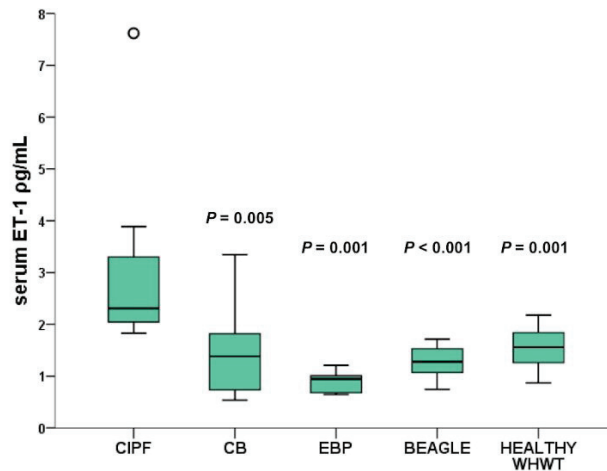


Figure 7 Serum endothelin-1 (ET-1) concentration in dogs with canine idiopathic pulmonary fibrosis (CIPF), chronic bronchitis (CB), eosinophilic bronchopneumopathy (EBP), healthy beagles and healthy West Highland white terriers (WHWTs). P values were as compared to CIPF dogs. The line indicates the median value and the boxes the interquartile range (IQ). The whiskers extend to 1.5 IQ. Values more than that are labelled as outliers and marked as dots.

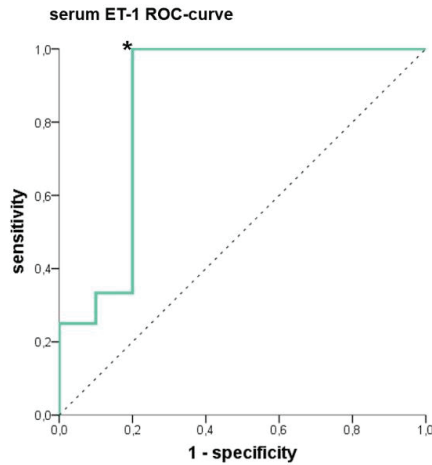


Figure 8 Receiver operating characteristic (ROC) curve of serum endothelin-1 (ET-1) concentration for differentiating canine idiopathic pulmonary fibrosis from chronic bronchitis. The optimal cut-off value of 1.8 pg/mL, indicated in the figure with an asterisk (*), yielded a sensitivity of 100% and a specificity of 85%. The area under the ROC curve was 0.86.

5.3.2 BRONCHOALVEOLAR LAVAGE FLUID ENDOTHELIN-1 CONCENTRATION

BALF ET-1 concentration was measured from 6 dogs with CIPF, 5 healthy WHWTs and 5 dogs with CB. In all CIPF dogs, the ET-1 concentration was measurable in BALF whereas in the dogs with CB and in healthy WHWTs, the BALF ET-1 concentration was below the detection limit of the assay (Fig. 9).

In dogs with CIPF, no correlation was found between BALF and serum ET-1 concentration ($P = 0.71$).

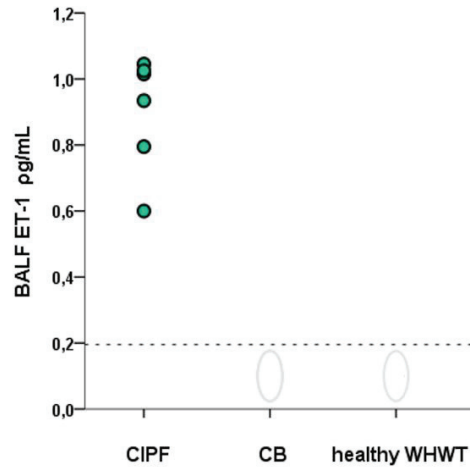


Figure 9 Dot plot of endothelin-1 (ET-1) concentration in bronchoalveolar lavage fluid (BALF) of dogs with canine idiopathic pulmonary fibrosis (CIPF), chronic bronchitis (CB) and healthy West Highland white terriers (WHWT). Scattered line represents the detection limit of the assay.

5.4 PIIINP ANALYSIS (STUDY III)

5.4.1 SERUM PIIINP CONCENTRATION

Serum PIIINP concentration was measured in 13 dogs with CIPF, nine dogs with CB, seven dogs with EBP and 13 healthy controls (all WHWTs). There was no statistically significant difference in serum PIIINP concentration between the groups (Fig. 10).

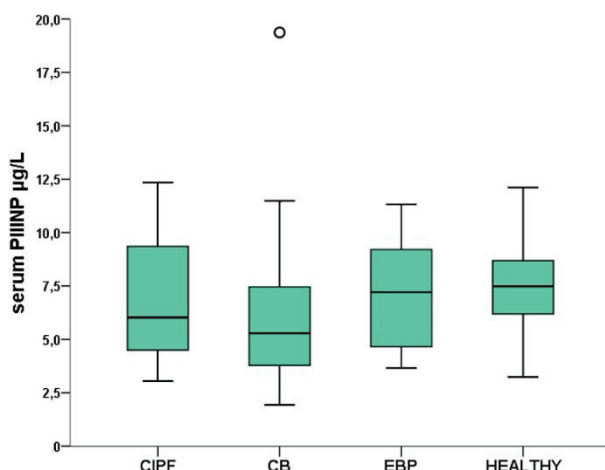


Figure 10 Serum procollagen type III amino terminal propeptide (PIIINP) concentration in dogs with canine idiopathic pulmonary fibrosis (CIPF), chronic bronchitis (CB), eosinophilic bronchopneumopathy (EBP) and in healthy dogs (WHWTs and beagles). There was no significant difference between the groups. The line indicates the median value, the boxes the interquartile range (IQ), the whiskers extend to 1.5 times IQ and values more than that are labelled as outliers and represented by dots.

5.4.2 BRONCHOALVEOLAR LAVAGE FLUID PIIINP CONCENTRATION

BALF PIIINP concentration was analysed from 13 dogs with CIPF, 19 dogs with CB, 13 dogs with EBP and 22 healthy control dogs (10 WHWTs and 12 beagles). The results and a comparison of BALF PIIINP concentration between CIPF and dogs with CB, EBP and healthy dogs are shown in Fig. 11.

BALF PIIINP concentration was significantly higher in dogs with EBP than in dogs with CB ($P = 0.003$) or healthy dogs ($P = 0.022$). No statistically significant differences were found regarding BALF PIIINP concentration between dogs with CIPF and dogs with EBP ($P = 0.812$), between dogs with CB and healthy controls ($P = 0.148$) or between healthy WHWTs and healthy beagles ($P = 0.473$).

No correlation was detected between BALF PIIINP and serum PIIINP concentration. In CIPF dogs, BALF PIIINP concentration was not correlated with PaO_2 , P(A-a)O_2 or duration of clinical signs.

The results of ROC curve analysis of BALF PIIINP concentration for distinguishing CIPF from CB is illustrated in Fig. 12.

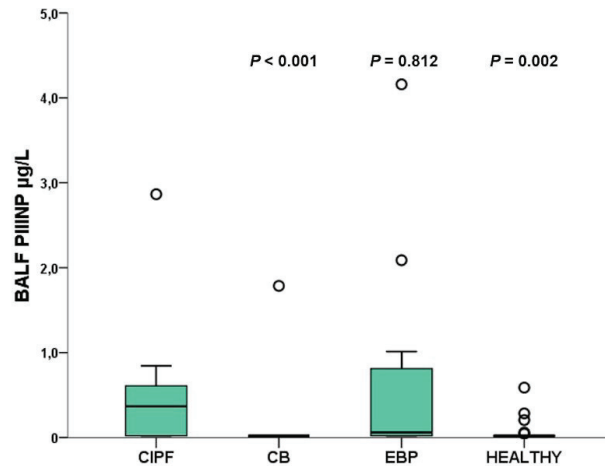


Figure 11 Bronchoalveolar lavage fluid (BALF) procollagen type III amino terminal propeptide (PIIINP) concentration in dogs with canine idiopathic pulmonary fibrosis (CIPF), chronic bronchitis (CB), eosinophilic bronchopneumopathy (EBP) and healthy dogs. *P* values were as compared to CIPF dogs. The line indicates the median value, the boxes the interquartile range (IQ), the whiskers extend to 1.5 times IQ and values more than that are labelled as outliers and marked as dots.

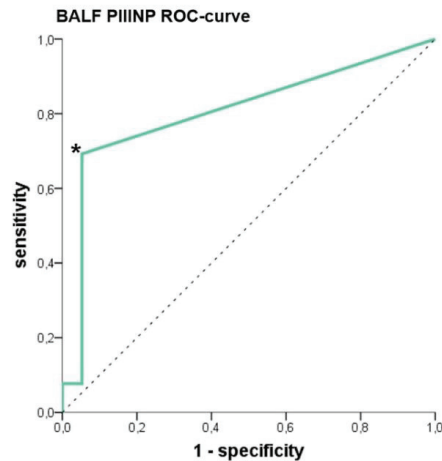


Figure 12 Receiver operating characteristic (ROC) curve of bronchoalveolar lavage fluid (BALF) procollagen type III amino terminal propeptide (PIIINP) concentration for differentiating canine idiopathic pulmonary fibrosis from chronic bronchitis. The optimal cut-off value 0.1 µg/L, indicated in the figure with an asterisk (*), yielded a sensitivity of 69% and a specificity of 95%. The area under the ROC curve was 0.81.

5.5 HISTOPATHOLOGY (IV)

The age of the WHWTs with CIPF from which the lung samples were obtained varied between 8 and 16 years (median 12 years). The control WHWTs were 2, 6 and 12 years of age.

5.5.1 INTERSTITIAL FIBROSIS

A pattern of diffuse and mature fibrosis was present in the alveolar walls of all the 18 WHWTs studied. This diffuse fibrosis was graded either as mild (11/18 dogs) or moderate (7/18 dogs). It was equally present in different lung lobes. Perivascular concentric accumulation of collagen was visible around pre- and post-capillary small vessels in all 18 WHWTs. None of the control dogs showed these lesions (Fig. 13A and B).

In addition to the mature diffuse fibrosis, the majority of the WHWTs (11/18 dogs) also had multifocal areas of fibrosis accentuation. In these areas the fibrosis was more severe and more cellular. The areas of accentuated fibrosis were located around the bronchioli or under the pleura (Fig. 13C). Honeycomb change, a cystic airspace lined by metaplastic bronchiolar epithelium, was detected in 7 of 11 WHWTs in the areas of fibrosis accentuation (Fig. 13D). Interstitial smooth muscle hyperplasia was present in 9 of these 11 WHWTs. Myofibroblasts were diffusely present in the fibrotic alveolar interstitium in the areas of accentuated fibrosis (Fig. 14A, B and C). Fibroblast foci, prominent interstitial foci of fibroblasts and myofibroblasts beneath the alveolar epithelium, were not found.

5.5.2 ALVEOLAR EPITHELIAL AND LUMINAL CHANGES

PCII atypia was present in 17 of 18 WHWTs with CIPF. In twelve of these dogs PCII hyperplasia was also detected (Fig. 15A). The presence of atypical PCII cells was limited to the multifocal areas of more severe fibrosis in 11 WHWTs. In the remaining 6 WHWTs with PCII atypia but no areas of severe fibrosis, atypical PCII cells were scattered in the alveolar septa throughout the lung samples. One dog with diffuse mature fibrosis and multifocal fibrosis accentuation areas had no epithelial changes.

Bronchiolar metaplasia of the alveolar epithelium, indicated by a strong expression of HMW-CK in epithelial cells covering the alveolar walls, was present in 11 of 18 WHWTs with CIPF in peribronchiolar fibrotic areas (Fig. 15B). Six of these WHWTs also had epithelial pseudostratification and squamous metaplasia.

A mild to moderate amount of foamy macrophages was detected in alveolar lumens in 13 of 18 WHWTs (Fig. 15C). This accumulation of macrophages was restricted to areas of disease accentuation. Intra-alveolar accumulation of proteinaceous material, manifested as a dense, eosinophilic and homogenous substance, was detected in 14 of 18 WHWTs with CIPF. This change was not related to areas of fibrosis accentuation, instead it was multifocally randomly distributed throughout the lung.

DAD presented as hyaline membranes lining the alveolar luminal walls, coinciding with hyperplastic PCII cells, scattered alveolar septal neutrophils, and alveolar septal congestion, was detected in 5 of 18 WHWTs with CIPF (Fig. 15D). DAD was limited to the areas of fibrosis accentuation. Consecutive luminal organising fibrosis was seen in these five WHWTs and also in two WHWTs with CIPF without DAD change.

Alveolar epithelial changes were not present in any of the control dogs. The two and six year old control dogs had mild alveolar oedema.

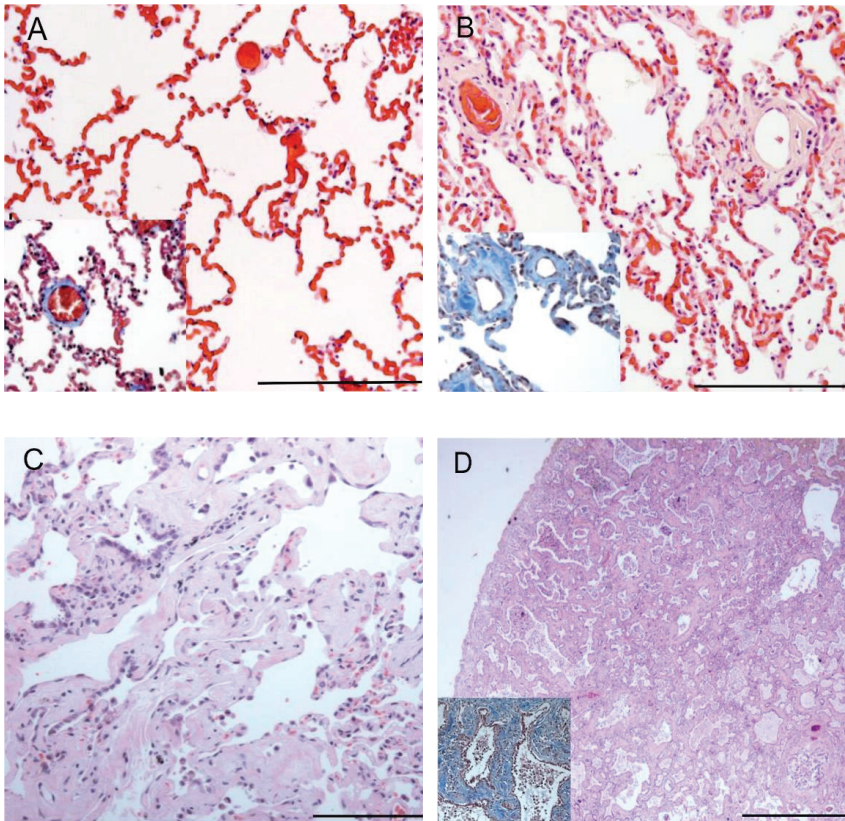


Figure 13 *Histopathological features of canine idiopathic pulmonary fibrosis in West Highland white terriers (WHWTs). (A) Lung histology of a healthy control WHWT. HE, Bar 200 μ m. Inset: Vessel of a healthy control WHWT. Masson's trichrome. (B) Mild diffuse mature interstitial fibrosis. HE. Bar, 200 μ m. Inset: Perivascular concentric fibrosis. Masson's trichrome (C) Focus of accentuated disease with severe interstitial fibrosis and pneumocyte type II hyperplasia. HE. Bar, 200 μ m. (D) Subpleural area of honeycombing and severe interstitial fibrosis. HE. Bar, 1 mm. Inset: Cystic fibrotic airspace within areas of honeycombing. Masson's trichrome. The images are taken from the publication IV.*

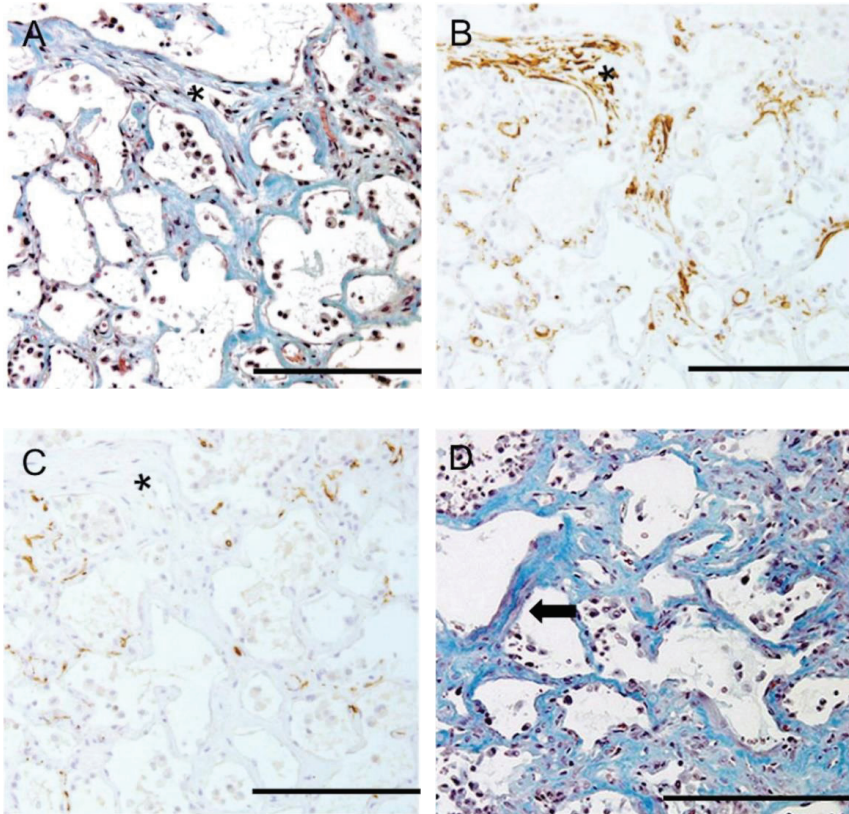


Figure 14 *Histopathological and immunohistochemical characteristics of the progressing fibrosis in canine idiopathic pulmonary fibrosis of West Highland white terriers. (A) Multifocal to coalescing areas of interstitial less mature fibrosis (asterisk). Masson's trichrome. Bar, 200 μ m. (B) Serial section of (A), showing SMA positivity in the area of more cellular fibrosis (asterisk). IHC SMA. Bar, 200 μ m. (C) Serial section of (A) and (B) negative for desmin in areas of SMA positivity (asterisk), consistent with presence of myofibroblasts in areas of progressing fibrosis. IHC desmin. Bar, 200 μ m. (D) Immature fibrosis organising along a central core of collagen-rich interstitial fibrosis (arrow) within the alveolar wall. Masson's trichrome. Bar, 200 μ m. The images are taken from the publication IV.*

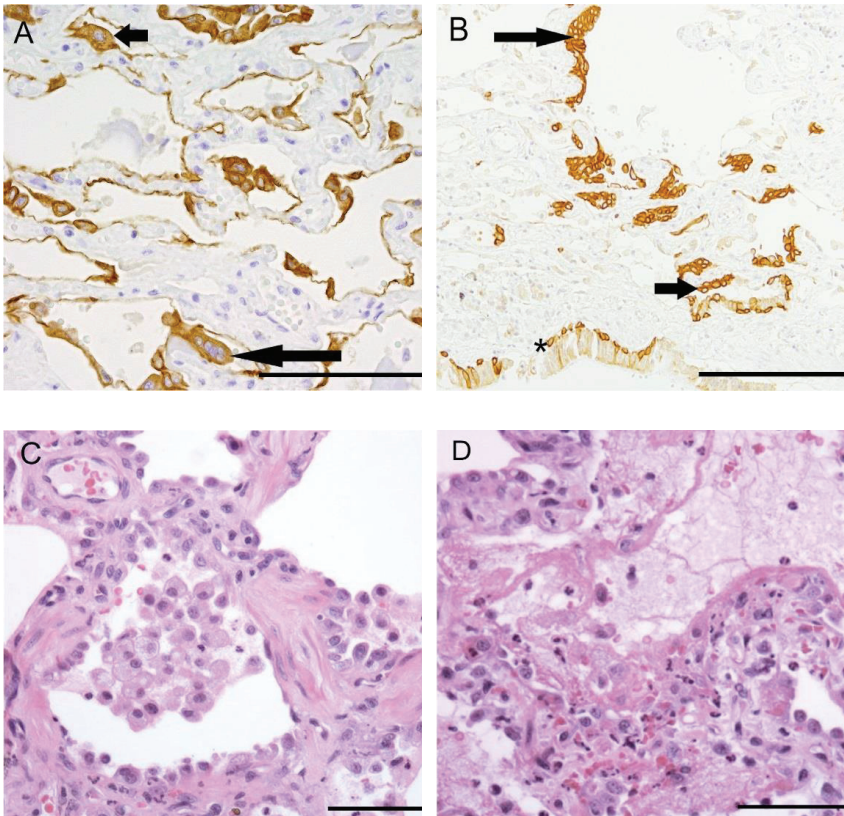


Figure 15 Alveolar epithelial and luminal changes of canine idiopathic pulmonary fibrosis in West Highland white terriers. (A) Pneumocyte type II (PCII) atypia. Broad-based (short arrow) and occasional multinucleated (long arrow) PCII cells lining the alveolar septa. IHC CK. Bar, 100 μ m. (B) Metaplasia of bronchiolar epithelium. HMW-CK positive hyperplastic (short arrow) and occasionally metaplastic (long arrow) epithelial cells on peribronchiolar alveolar septa. Basal bronchiolar cells expressing HMW-CK in a normal bronchiole are shown by an asterisk. IHC HMW-CK, Bar 200 μ m. (C) Interstitial smooth muscle metaplasia, desquamating alveolar macrophages and PCII hyperplasia within the severe fibrotic areas. HE. Bar, 20 μ m. (D) Acute alveolar damage with hyaline membrane formation. HE. Bar, 20 μ m. The images C and D are taken from the publication IV.

5.5.3 INFLAMMATION

In addition to interstitial fibrosis, all WHWTs with CIPF showed interstitial lymphoplasmacytic inflammation with few macrophages. The inflammation was considered mild in 13 of 18 WHWTs and moderate in 5 of 18 WHWTs. Eight WHWTs with CIPF had concurrent mild to moderate lymphoplasmacytic bronchitis and three WHWTs had multifocal, mild, chronic fibrosing pleuritis.

The oldest control WHWT had multifocal mild peribronchiolar lymphoplasmacytic inflammation. The two other control WHWTs had no inflammatory changes in their lung.

5.5.4 OTHER FINDINGS

Interstitial anthracosis was seen in all WHWTs with CIPF and in all control WHWTs. It was described as mild to moderate, multifocal, peribronchiolar and perivascular in 16 WHWTs with CIPF and in control WHWTs, and severe in 2 WHWTs with CIPF.

Thrombosis of pulmonary vessels with partial organisation of thrombi was detected in two WHWTs with CIPF. One of those dogs had moderate and one had severe pulmonary changes. No vascular changes were detected in the control dogs.

5.5.5 DISTRIBUTION OF THE MOST SEVERE LESIONS

Histological samples from all lung lobes were obtained from 9 of the 18 WHWTs with CIPF. Table 8 presents a comparison of the severity of the histopathological lesions between different lung lobes in these nine dogs. Severe multifocally accentuated lesions, indicated by severe interstitial fibrosis and/or profound alveolar epithelial and luminal changes and inflammation, were present in the caudal lobes in three dogs and in the cranial lobes in one dog with mild diffuse fibrosis. In all five dogs with moderate diffuse fibrosis, both cranial and caudal lung lobes showed multifocally accentuated lesions.

Table 8 *Comparison of the severity of the histopathological lesions between different lung lobes in nine WHWTs with CIPF.*

Dog No.	Age years	Diffuse fibrosis	Multifocally accentuated fibrosis	Cranial lobes		Caudal lobes		Middle lobe	Accessory lobe
				left	right	left	right		
1	16	mild	not present	more severe	less severe	less severe	less severe	less severe	less severe
2	13	mild	moderate	less severe	less severe	more severe	more severe	less severe	less severe
3	15	mild	moderate	less severe	less severe	more severe	more severe	more severe	more severe
4	14	mild	severe	less severe	less severe	more severe	more severe	NA	NA
5	12	moderate	not present	equal	equal	equal	equal	NA	NA
6	8	moderate	not present	equal	equal	equal	equal	equal	equal
7	13	moderate	severe	equal	equal	equal	equal	equal	equal
8	12	moderate	severe	equal	equal	equal	equal	NA	NA
9	12	moderate	severe	equal	equal	equal	equal	NA	NA

CIPF, canine idiopathic pulmonary fibrosis; NA, not applicable; WHWT, West Highland white terrier.

5.5.6 COMPARISON WITH HUMAN USUAL INTERSTITIAL PNEUMONIA AND NONSPECIFIC INTERSTITIAL PNEUMONIA

Table 9 summarises the histopathological classification criteria of human UIP and NSIP (American Thoracic Society, European Respiratory Society, 2002; Katzenstein et al., 2008; Raghu et al., 2011), and their presence or absence in the lungs of the WHWTs with CIPF.

In 7 of the 18 WHWTs with CIPF, the histopathological findings resembled fibrosing NSIP. The interstitial fibrosis was relatively diffuse, it was of variable degree, alveolar architecture was preserved and there was mild to moderate interstitial inflammation (Fig. 13B compared with Figs 16A and C). In another seven dogs, additional patchy areas of honeycombing, comparable to UIP, were seen (Fig. 13D compared with Fig. 16B). The remaining four WHWTs with CIPF had multifocal accentuation of fibrosis without architectural distortion or honeycombing.

No fibroblast foci were detected in WHWTs with CIPF. Fibroblast foci are seen in human UIP (Fig. 16D). The alveolar epithelial lesions were more diffuse and prominent in the WHWTs, but comparable in morphology to the epithelial changes in human NSIP. The presence of bronchiolar metaplasia of peribronchiolar fibrotic alveolar septa was a dominant feature of CIPF in WHWTs and in human UIP, but not prominent in human NSIP.

5 Results

Table 9 *Comparison between the main histological criteria required for the diagnoses of UIP or NSIP in humans and the findings in CIPF in WHWTs.*

Criteria	UIP	CIPF	NSIP (fibrosing)
Pattern	Patchy, subpleural or paraseptal	Diffuse with subpleural/ peribronchiolar accentuation	Relatively diffuse
Interstitial fibrosis	Marked, distorting, replacing alveolar tissue	Mild to marked, not obliterating alveolar architecture	Variable degree
Honeycombing	Yes	Yes	Not characteristic
Fibroblastic foci	Yes	No	Absent or inconspicuous
Interstitial inflammation	Minimal, mild	Mild to moderate	Mild to moderate
SM hyperplasia	Yes	Yes	Not characteristic
PCII	Hyperplasia	Hyperplasia, atypia	Hyperplasia in areas of inflammation
Bronchiolar epithelium	Bronchiolar metaplasia of alveolar epithelium	Bronchiolar metaplasia of alveolar epithelium	Not recorded

CIPF, canine idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; PCII, type II pneumocyte; SM, smooth muscle; UIP, usual interstitial pneumonia; WHWT, West Highland white terrier.

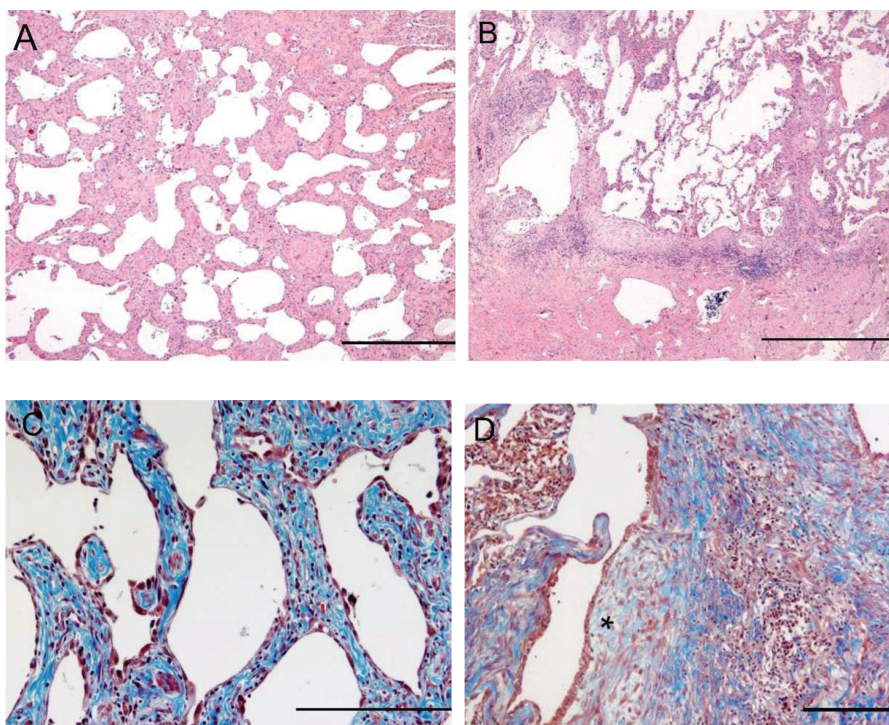


Figure 16 *Characteristic lesions of human nonspecific interstitial pneumonia (NSIP) and human usual interstitial pneumonia (UIP). (A) Diffuse interstitial fibrosis pattern of NSIP. HE. Bar, 1 mm. (B) A subpleural area of severe distorting fibrosis in UIP. HE. Bar, 1 mm. (C) Temporally homogenous interstitial fibrosis in NSIP. Masson's trichrome. Bar, 200 μ m. (D) Temporally heterogeneous fibrosis, with fibroblast foci (asterisk) in UIP. Masson's trichrome. Bar, 1 mm. The images are taken from the publication IV.*

6 DISCUSSION

6.1 CIPF – CLINICAL DISEASE

Our goal for the study was to further define the clinicopathological and diagnostic features in dogs with CIPF. The study was prospective and included a control group of healthy WHWTs. In this thesis summary, the results for 15 dogs with CIPF are presented, which is three more than in Study I. All the dogs with CIPF had histopathological confirmation of the diagnosis.

The signalment, clinical signs and the findings from physical examinations of the dogs in our study with CIPF were in line with those reported previously (Corcoran et al., 1999a). The affected dogs had a mean age of 12 years, and no predisposition for sex was detected. The mean duration of clinical signs was 14 months, but it varied considerably (range 2-29 months). The most common clinical sign was the combination of cough and exercise intolerance, but not all the dogs coughed. The reason for the cough in CIPF is unclear. The bronchial changes present in many CIPF dogs may contribute to the cough, but are not likely to be the only explanation. We also detected bronchial changes in dogs which did not cough and, on the other hand, in one coughing dog the airway changes were mild, consisting only of mucosal irregularity and grade I tracheal collapse. Human patients with IPF have an enhanced cough reflex sensitivity that is hypothesised to be due to upregulation of the sensory nerves of the respiratory tract (Hope-Gill et al., 2003). It is unknown whether this also exists in dogs.

Bilateral, inspiratory Velcro crackles were heard in auscultation in 12 of the 15 dogs with CIPF, but in none of the healthy controls. This strengthens the previous conclusion that Velcro crackles are associated with CIPF (Corcoran et al., 1999a; Lobetti et al., 2001) as they are with human IPF (Raghu et al., 2011). In human patients, Velcro crackles can be the first sign of IPF and can help in detecting the disease early (Cottin and Cordier, 2012). Interestingly in this study, the most severely affected dog was one of the three in which Velcro crackles were not heard. Our finding suggests that an absence of Velcro crackles cannot be used to rule out even severe CIPF. An explanation for the absence of the crackles could be the shallow tachypnoeic breathing present in the severely affected dog. In humans, slow breathing from near-residual lung volume helps in detecting the sound (Kraman, 1986). Since regulation of the breathing pattern in dogs is not possible in the same way as in humans, inducing deep breaths by temporarily closing the nostrils could be used to help the detection of crackles.

As we expected hypoxemia to be the key clinical consequence of CIPF, we quantified the degree of hypoxemia in our dogs affected by the disease. Arterial blood gas analysis revealed surprisingly low PaO₂ and high P(A-a)O₂ values in CIPF dogs considering that most dogs were bright and alert. The slow progression of the disease makes it possible for the dogs to become accustomed to the lowering oxygen values. Retention of PaCO₂ was not detected in any of the dogs. These findings are in line with human IPF: the average patient is hypoxemic at rest, P(A-a)O₂ is almost always abnormal and alveolar hypoventilation leading to hypercapnia is rare (Crystal et al., 1976; Nava and Rubini, 1999).

CIPF was not associated with changes in serum biochemistry or haematology. Only one of our CIPF dogs had a haematocrit above the reference range (61%). The dog was not dehydrated, it had a PaO₂ of 42 mmHg and it had had clinical signs for a year. An increase in haematocrit (59%) was also noted in one of the control dogs. The lack of polycythaemia in the other dogs with CIPF is an interesting finding. In humans, polycythaemia is not linked to IPF as it is to other chronic hypoxemic diseases. The reason for this is not known (Crystal et al., 1976; American Thoracic Society, European Respiratory Society, 2000). To our knowledge, the only previously reported polycythaemic CIPF dogs were two young Staffordshire bull terriers (haematocrit 71% and 72%) in the study of Lobetti et al (2001). These findings suggest that despite suffering from chronic hypoxemia, polycythaemia is not commonly observed in WHWTs with CIPF.

In accordance to previous reports (Corcoran et al., 1999a; Johnson et al., 2005), bronchointerstitial lung pattern was the most commonly detected change in thoracic radiographs in our dogs with CIPF. These radiographic signs associated with CIPF are nonspecific. We also observed moderate to severe bronchointerstitial lung pattern in all dogs with CB, and approximately half the dogs with EBP had both bronchial and interstitial opacities. Alveolar opacities were seen in three CIPF dogs. In one, HRCT was performed and no such finding could be observed. This suggests that alveolar opacity in radiographs might be at least partly explained by poor inflation of the lung. In thoracic radiographs of human patients with IPF, honeycombing becomes evident when disease progresses (Crystal et al., 1976). To our knowledge, honeycomb change has not been described in radiographs of dogs with CIPF.

HRCT is a much more sensitive imaging method for detecting changes in the lung parenchyma than thoracic radiographs. HRCT is gaining more and more ground in veterinary medicine and is crucial in diagnosing human IPF (Raghu et al., 2011). The most common finding in our dogs with CIPF was GGO, described as a hazy increase in overall lung density. Similar to the findings of Johnson et al. (2005), GGO was visible in all our dogs with CIPF investigated by HRCT. This contrasts to HRCT findings of human IPF. In humans, the presence of GGO points more towards an alternative diagnosis, such as NSIP, whereas honeycombing, traction bronchiectasis, coarse reticulation and architectural distortion are characteristic of IPF (Gotway et al., 2007). Here, reticular opacities were seen in all dogs with CIPF but in none of the controls. Traction bronchiectasis was present in five and honeycombing in two dogs. Johnson et al. (2005) suggested that traction bronchiectasis and honeycombing appear when CIPF is already severe, however, in our study both findings were present in the least affected dog.

In bronchoscopy, we detected signs of airway involvement in all CIPF dogs, CB dogs and EBP dogs. A variety of changes was described in each group. This indicates that bronchoscopic findings commonly associated with airway disease cannot be used to rule out CIPF. Mucosal irregularity, which was present in all dogs with CIPF, and approximately every second dog with CB and EBP, was also noted in the majority of the healthy control WHWTs. It could be at least partly explained by the old age of the dogs (Mercier et al., 2011). An increased amount of bronchial mucus is one of the key features of CB (Pirie and Wheeldon, 1976) and commonly reported in EBP (Rajamäki et al., 2002). It seemed to be more often a concern in dogs with CB (detected in 14 of the 22 dogs) and dogs with EBP

(12 of 15 dogs) than in dogs with CIPF (3 of 9 dogs). Instead, tracheal collapse was more commonly observed in WHWTs with CIPF (6 of 9 dogs) than in other dogs (3 of 22 CB dogs, none of the EBP dogs). Corcoran et al. (2011) also reported that tracheal collapse was common in WHWTs with CIPF. This might contribute to the clinical presentation of CIPF dogs, at least if the collapse is severe, however, the true significance of this finding and its possible relationship, if any, to the underlying ILD is unclear. Nevertheless, we also detected mild tracheal collapse in three of the nine healthy control WHWTs. The WHWT has not been previously reported to be among the breeds predisposed to tracheal collapse (Della Maggiore, 2014). Our findings indicate that airway changes are common in dogs with CIPF. We do not know whether the changes occur secondarily to CIPF or as an individual phenomenon. Despite the degree of airway changes, all our CIPF dogs had a similar histopathologic picture of interstitial fibrosis.

Previously, mainly normal to moderately increased BALF cellularities have been described in dogs with CIPF (Corcoran et al., 1999a; Corcoran et al., 2011). However, BALF cellularities have not been compared between dogs with CIPF and healthy dogs. We elected to have a healthy control group of the same breed in order to exclude the possibility that breed would influence our results. So far, no studies comparing BALF cellularities between different dog breeds have been conducted. In our study, the dogs with CIPF had an increase in BALF TCC due to increased numbers of macrophages, neutrophils and mast cells. On histopathological examination of lung tissue, intra-alveolar accumulation of macrophages was evident in many dogs, reflecting the role of macrophages in CIPF pathology. The increase in TCC is likely a consequence of the ILD rather than solely a secondary bronchial reaction to coughing, as it was also increased in those dogs that had not been coughing. In Study I, the only change in relative differential cell counts was detected in the lymphocyte percentage, which was lower in dogs with CIPF than in healthy WHWTs. As we included more dogs in the thesis summary than in Study I, changes in eosinophilic percentage (lower in CIPF than in healthy) and mast cell percentage (higher in CIPF than in healthy) also reached statistical significance. We correlated BALF TCC, individual and differential cell counts with PaO₂ and detected only weak correlations, which may not be of clinical value. In human IPF, TCC is increased and BALF neutrophilia and mild or moderate eosinophilia are described. Lack of lymphocytosis supports the IPF diagnosis. In NSIP, BALF lymphocytosis is typical (Domagała-Kulawik et al., 2012; Meyer et al., 2012).

6.2 CIPF BIOMARKERS

Given the challenges to diagnosing CIPF in dogs, one of the aims of this thesis was to identify non-invasive biomarkers of fibrosis which could help in detecting the disease. In this thesis we investigated whether ET-1 or PIINP could be useful in differentiating CIPF from other chronic respiratory diseases in dogs. We measured ET-1 and PIINP concentration in both serum and BALF of dogs with CIPF, CB, EBP as well as in two groups of healthy control dogs.

Obtaining BALF requires anaesthesia, which cannot be performed on dogs with very advanced lung disease. The advantage of a BALF marker is, however, that it may provide a

more accurate indication of lung disease than serum markers, which are more likely influenced by various processes unrelated to pulmonary pathology. A serum biomarker is easily obtained and therefore more practical to use.

6.2.1 ENDOTHELIN-1

Serum ET-1 concentration was elevated in dogs with CIPF when compared with dogs with CB, EBP or healthy dogs. Additionally, BALF ET-1 was measured in dogs with CIPF and CB to study whether it could differentiate these two diseases. In BALF, ET-1 concentration was above the detection threshold of the test only in dogs with CIPF. These findings are in agreement with findings in human IPF in which elevated ET-1 concentration is found in plasma, BALF and lung tissue (Giaid et al., 1993; Sofia et al., 1993; Ugucioni et al., 1995). ET-1 has a well established role in the fibrogenesis of human IPF. Among other things, it acts synergistically with other fibrosis mediators, such as TGF- β , and participates in the recruitment of fibroproliferative cells, induces production of collagen and reduces collagenase activity; events which ultimately lead to excess collagen deposition in the lung (Swigris and Brown, 2010). Our results suggest that ET-1 may also be implicated in the pathogenesis of CIPF.

In humans, ET-1 concentration is known to increase with age (Komatsumoto and Nara, 1995). As our dogs with CIPF were older than dogs with CB, EBP or healthy dogs, we wanted to investigate the possible influence of age on the ET-1 concentration. A covariate analysis did not show that age would affect ET-1 values. This is in line with a previous study of 76 healthy dogs in which plasma ET-1 concentration was not found to correlate with age (Tessier-Vetzel et al., 2006).

We included both healthy WHWTs and healthy beagles as healthy control dogs. There was no difference in serum ET-1 concentration between these two groups, excluding a possible breed influence on ET-1 results.

No elevation was detected in serum ET-1 concentration in dogs with CB or EBP when compared with healthy dogs. Previously, high plasma ET-1 concentration was detected in a group of dogs with respiratory disease when compared to healthy dogs. Dogs with severe respiratory disease had higher ET-1 values than dogs with occasional coughing, however, the study did not separate the different respiratory diseases (Tessier-Vetzel et al., 2006). In humans, in addition to IPF, an increase in ET-1 is also associated with other lung diseases. In asthma, serum and BALF ET-1 concentration is elevated and ET-1 is thought to contribute to bronchoconstriction typical for the disease (Sofia et al., 1993; Gawlik et al., 2006). Asthma is not recognised as such in dogs, but EBP shares some of its features, such as airway eosinophilia and T helper 2 dominant immune response (Clercx and Peeters, 2007). It seems that ET-1 does not have a similar importance in the pathology of EBP as it has in human asthma. In human patients with COPD, it has been suggested that ET-1 is involved in airway obstruction and the exaggerated sputum production characteristic of the disease (Roland et al., 2001). Both elevated and normal ET-1 levels have been reported in human patients with COPD, however (Chalmers et al., 1999; Reichenberger et al., 2001;

Roland et al., 2001). Canine CB is often compared to human COPD, although these two diseases can have quite different clinical pictures. ET-1 appears not to be implicated in the pathogenesis of CB in dogs.

A ROC curve analysis confirmed that serum ET-1 concentration can be used to differentiate CIPF from CB with good accuracy. The optimal cut off value yielded an excellent sensitivity of 100% and a good specificity of 85%. Previous studies have shown that overt heart failure and PH increase ET-1 concentration in dogs (Prošek et al., 2004; Tessier-Vetzel et al., 2006), and therefore, an echocardiographic examination aimed specifically at detecting PH should be performed if ET-1 measurements are to be used as an adjunctive method for diagnosing CIPF.

ET-1 being measurable from serum is a clear advantage for its use as a biomarker of CIPF, however, we detected no correlation between serum ET-1 concentration and the severity or duration of CIPF. In addition, Lilja-Maula et al. (2014a) showed that ET-1 has no prognostic value in CIPF. ET-1 could therefore be useful as a diagnostic biomarker for CIPF, but not used to monitor the progression of the disease.

6.2.2 PIIINP

Serum PIIINP concentration does not seem to be useful in assessing lung diseases in dogs. There was no difference in serum PIIINP concentration between dogs with CIPF, CB, EBP or healthy dogs, nor did it correlate with BALF PIIINP concentration. This is in accordance with an earlier study conducted in dogs suffering from chronic bronchopneumopathy (Schuller et al., 2006), but contradicts some studies in humans (Kirk et al., 1984; Low et al., 1992; Harrison et al., 1993; Lammi et al., 1999).

CIPF instead caused an elevation in BALF PIIINP concentration. This agrees with findings in human IPF (Bjermer et al., 1989; Harrison et al., 1993; Hiwatari et al., 1997; Lammi et al., 1999). High BALF PIIINP is thought to signify active collagen type III synthesis in the lung, and therefore, BALF PIIINP could be a marker of disease activity rather than indicate the severity of the disease (Lammi et al., 1999).

We used oxygen values as markers of disease severity in our CIPF dogs, as did Lilja-Maula et al. (2014a) in their study, and investigated whether BALF PIIINP correlated with these or with the duration of clinical signs. No correlation was detected. Studies in which BALF PIIINP was correlated with the severity of IPF in humans have offered variable results, however, high BALF PIIINP concentration has repeatedly been associated with more rapid disease progression and poorer prognosis in human IPF (Bjermer et al., 1989; Low et al., 1992; Lammi et al., 1999). No studies have evaluated the use of BALF PIIINP concentration as a prognostic marker in dogs with CIPF.

Elevated BALF PIIINP concentration is not specific to CIPF. In addition to dogs with CIPF, dogs with EBP also had higher BALF PIIINP concentration than dogs with CB or healthy dogs in our study. This finding confirms the results of a previous study, in which an elevation of BALF PIIINP was detected in 15 dogs with chronic bronchopneumopathy, 13 of which had EBP (Schuller et al., 2006). It is likely that elevated BALF PIIINP concentration is caused by secondary fibrotic changes due to chronic eosinophilic

inflammation of the lungs. Based on the histology of bronchial biopsies, fibrotic changes are reported to accompany severe bronchial inflammation in EBP dogs (Clercx et al., 2000). Although BALF PIIINP is elevated also in dogs with EBP, it does not raise a concern for its use as a biomarker of CIPF. CIPF can be easily differentiated from EBP by BALF cytology analysis: marked eosinophilia is a requirement for EBP diagnosis but is not characteristic of CIPF.

Differentiating CIPF from CB is a greater challenge. Both diseases share clinical and diagnostic features, and bronchoscopic findings suggestive of CB also occur in dogs with CIPF. Interestingly, BALF PIIINP concentration was not elevated in dogs with CB in our study. This finding agrees with a previous study in which BALF PIIINP concentration did not rise after experimentally inducing bronchial inflammation in dogs (Lonneux et al., 2009). In humans, levels of PIIINP are increased in airways of patients with COPD (Harju et al., 2010). We further assessed the ability of BALF PIIINP to differentiate CIPF from CB by using ROC curve analysis. The AUC of 0.81 suggests that BALF PIIINP could be used to differentiate these two diseases with moderate accuracy (Swets, 1988). Investigating the BALF PIIINP concentration could therefore be a useful addition to other diagnostic tests for differentiating these two diseases, although PIIINP being elevated only in BALF and not in serum is a disadvantage which makes it applicable only in limited circumstances.

6.3 CIPF – HISTOPATHOLOGICAL ASPECTS

The histopathological features of CIPF have been unclear. Consequently, it has not been known whether there is resemblance between CIPF and human disease and if so, to what extent. Previous studies of CIPF have included only a limited number of dogs, and perhaps for this reason the results have been contradictory. We performed a comprehensive study of the histopathological features of CIPF by investigating lung samples from 18 WHWTs with the disease using routine staining, collagen staining, and immunohistochemical techniques, and provided a comparison with human UIP and NSIP.

Two different kinds of interstitial fibrosis were detected in WHWTs with CIPF. A pattern of diffuse and mature homogenous fibrosis was present in all dogs, and a multifocally accentuated pattern of severe, less mature fibrosis was also detected in the majority of the dogs. The diffuse fibrosis varied from mild to moderate in severity and was equally distributed throughout the lung. It did not lead to architectural distortion. A similar type of interstitial fibrosis was described in six dogs with CIPF by Norris et al. (2005). This diffuse, mature and uniform-appearing interstitial fibrosis, detected in all WHWTs in our study, closely resembles the fibrosis pattern of human NSIP (American Thoracic Society, European Respiratory Society, 2002; Katzenstein et al., 2008). As the diffuse fibrosis was mature and present in all dogs, it might represent an early lesion of CIPF. It is not known what triggers the formation of diffuse fibrosis in WHWTs. Interstitial fibrosis with concentric deposition of collagen around small vessels, as detected here and also by Norris et al. (2005), can occur as a consequence of PH. PH is a common complication of CIPF and we suspected it in several dogs, however, it is not likely to be the only reason, as we did not consistently find other histopathological findings indicating PH, such as pulmonary arterial

hypertrophy (Van Vleet and Ferrans, 2007). In humans, the histopathological pattern of NSIP is commonly seen as a lung manifestation of systemic connective tissue diseases (Travis et al., 2013). A disease with systemic increase in collagen deposition has not been described in dogs. TGF- β , a key mediator of fibrosis in humans, was recently shown to be involved in the fibrogenesis of CIPF (Lilja-Maula et al., 2014c). An increased serum concentration of TGF- β , detected both in healthy and sick WHWTs, could be one of the explanatory factors predisposing the dogs to fibrosis (Krafft et al., 2014).

In addition to diffuse fibrosis, we observed multifocal areas of more severe, more cellular and less mature fibrosis. These areas were accompanied by profound alveolar epithelial and luminal changes, smooth muscle hyperplasia and occasional honeycombing. This change bears close resemblance to human UIP (American Thoracic Society, European Respiratory Society, 2002; Raghu et al., 2011). Multifocally accentuated fibrosis was also described in dogs with CIPF by Corcoran et al. (1999a) and Erikson et al. (2009). In our study, the areas of more severe fibrosis were located either around the bronchioli or under the pleura. In human UIP the areas of architectural distortion appear patchy and are located subpleurally and paraseptally, but not peribronchiolarly (Katzenstein et al., 2008). Paraseptal location refers to sites near the interlobular connective tissue septae which are absent in canine lungs. One explanation for the subpleural location of UIP changes might be stretching of the distal lung due to reduced lung compliance (Günther et al., 2012). Hunninghake and Schwarz (2007) suggested another explanation by speculating that the peripheral parts might be more accessible to injury or less prone to normal repair. It remains to be investigated whether these hypotheses are true in dogs. The peribronchiolar location of areas of accentuated fibrosis indicates that an additional inhaled etiological factor could be involved in the pathogenesis of CIPF.

PCII hyperplasia and atypia were a consistent finding in CIPF based on our results. This is in line with changes characteristic of UIP and may indicate a repetitive damage to the alveolar epithelium. In UIP, chronic injury to the alveolar epithelium and excessive apoptosis of alveolar epithelial cells may lead to exhaustion of the alveolar epithelial regenerative potential. This in turn could cause alveolar epithelial cells to lose control over mesenchymal cells leading to fibroblast proliferation and an accumulation of collagen (Günther et al., 2012).

In addition to PCII changes, the majority of the dogs with CIPF in our study had bronchiolar metaplasia of the alveolar epithelium. This finding is also described in UIP. Bronchiolisation of the alveoli could arise from migration and differentiation of bronchial progenitor cells into the alveoli in an attempt to repair the damaged alveolar epithelium (Betsuyaku et al., 2000). Recently, in addition to the alveolar epithelium, bronchiolar epithelium of the distal airways has also been suggested to contribute to the pathogenesis of UIP in humans. This idea is supported by abnormal bronchiolar proliferation present in UIP, other histological evidence of small airway involvement and recent genetic studies showing mucin 5B gene promoter polymorphism as a risk factor for IPF (Chilosi et al., 2002; Figueira de Mello et al., 2010; Fingerlin et al., 2013; Seibold et al., 2013).

Fibroblast foci, a hallmark of human UIP, were not seen in any of the WHWTs with CIPF. In UIP, fibroblast foci signify areas of active fibrosis and temporal heterogeneity of the fibrosis process, in NSIP the fibroblast foci are usually absent (Katzenstein et al., 2008).

It is not known, however, why fibroblasts and myofibroblasts organise into such foci in UIP. Myofibroblasts are active cells which secrete excess amounts of extracellular matrix components and play an important role in creating a profibrotic milieu (King Jr et al., 2011b). Interestingly, we could identify scattered myofibroblasts in the interstitium in areas of more cellular fibrosis. This finding suggests that although not organised in specific bundles, myofibroblasts also participate in fibrogenesis in CIPF.

In addition to proliferating fibroblasts and the production of collagen, other mechanisms may also contribute to the development of interstitial fibrosis. Katzenstein (1985) described a fibrosis process in which intra-alveolar exudates were incorporated into the alveolar wall. This was found to take place in lungs of patients with acute interstitial pneumonia characterised histopathologically by DAD. PCII cells proliferated on top of intraluminal cellular debris located near the denuded alveolar basement membrane (Katzenstein, 1985). We detected DAD and organising fibrosis along the alveolar luminal lining in several dogs with CIPF. Areas of DAD co-localised within areas of more severe fibrosis. We suggest that incorporation of alveolar exudates into alveolar septa could also contribute to the development of fibrosis in dogs with CIPF.

We did not detect severe fibrotic scarring obliterating larger areas of the alveolar architecture. Even in the most affected areas, alveolar spaces could be identified between severely fibrotic alveolar walls. This differs from human UIP, in which profound scarring can lead to complete loss of alveolar structural design (Katzenstein et al., 2008).

Based on our findings, inflammation is not prominent in CIPF. We detected only mild to moderate lymphoplasmacytic interstitial inflammation in our dogs. Norris et al. (2005) described an inflammation of the same cell type but of more varying degree. Inflammation is not a prominent feature of UIP either. Our finding supports the hypothesis that ongoing inflammation is an unlikely cause for the progressive fibrosis.

Alveolar proteinosis was common in WHWTs with CIPF. Alveolar proteinosis results when surfactant accumulates into the alveolar lumens. It can result from either increased production or modification of surfactant by PCII cells or decreased surfactant clearance by alveolar macrophages. We speculate that both the PCII cells, which were morphologically abnormal, as well as the intra-alveolar macrophages which we could frequently detect, could be involved in the development of alveolar proteinosis in CIPF. As a primary disease, alveolar proteinosis is rare and only a few case reports have been published involving dogs (Jefferies et al., 1987; Silverstein et al., 2000; Cummings et al., 2013). Alveolar proteinosis is a chronic progressive ILD and can be considered one of the differential diagnoses of CIPF, however, affected dogs have been considerably younger. Alveolar proteinosis can occur secondarily to various lung disorders, and it has been associated with the inhalation of irritant substances in humans and in experimental animal models (Borie et al., 2011). This could support the hypothesis that an inhaled irritant would be involved in the pathogenesis of CIPF. In human patients with alveolar proteinosis, interstitial fibrosis can sometimes be detected in histopathology (Arbiser et al., 2003). Arbiser et al. suggested that an accumulation of surfactant could promote fibrosis. It is not known whether it contributes to the diffuse interstitial fibrosis typical of CIPF.

6.4 LIMITATIONS OF THE STUDY

We recruited 15 dogs with CIPF into our studies. The small number of dogs decreased the power of our statistical analyses. The number of CIPF dogs undergoing HRCT (7 dogs) was especially small. This should be taken into account when interpreting HRCT findings.

Although CIPF can be considered a disease of WHWTs, we decided to add one Scottish terrier to our study population. It can be speculated whether this brought additional value by increasing the case number or whether it was confusing. The results of the clinicopathological, diagnostic imaging and biomarker examinations obtained from the Scottish terrier were in line with those of the WHWTs with CIPF participating in the study. It is not known whether CIPF is exactly the same disease in different breeds of dogs. Because CIPF is not common, recruiting cases for research is challenging. Scottish terriers and WHWTs originate from the same Scottish dog ancestors and are therefore likely to be closely related. We argue that adding a Scottish terrier adds as much value to our studies as adding a WHWT.

We were able to include a control group of 14 healthy WHWTs in our studies. To confirm their health status, the majority of the dogs underwent very thorough examinations including arterial blood gas analysis, HRCT, bronchoscopy and BAL, and showed no signs of lung disease, however, as histopathology was only obtained from one of the dogs at a later date, a subclinical respiratory disease cannot be completely excluded. Whether a subclinical respiratory disease would explain the overlap in BALF PIIINP concentration between CIPF and healthy dogs, can only be speculated.

PH is common in dogs with CIPF, affecting more than 40% of the diseased dogs (Schober and Baade, 2006). We suspected it in 7 of the 13 dogs undergoing echocardiography. Those dogs showed right-sided ventricular dilatation and hypertrophy without primary cardiac diseases explaining the changes, however, as the pressure gradients across tricuspid valve were not calculated, we were not able to confirm the presence of PH in our dogs. Concurrent PH can significantly increase ET-1 concentration (Tessier-Vetzel et al., 2006). We therefore cannot exclude an effect from PH on the increased ET-1 results obtained from our dogs with CIPF. Serum ET-1 concentration was not different between the dogs suspected to have PH and those with normal echocardiographic examination, however. The dog with the highest serum ET-1 concentration did not have any findings indicating PH.

This study was conducted as a bicentre study. Examinations took place at the Veterinary Teaching Hospitals of the Universities of Helsinki and Liège. For this reason, we used two different BAL collection protocols. In one protocol, a weight-adjusted amount of lavage fluid was used (2 mL/kg divided in two aliquots), and in the other protocol, a fixed amount of lavage fluid was instilled (60 ml divided into three aliquots). This might have affected the dilution factor and the amount of recovered epithelial lining fluid. Melamies et al. showed (2011) that the weight-adjusted protocol leads to less variance in epithelial lining fluid recovery than a fixed-amount technique, however, the difference was small. It is unlikely that the different BALF protocols would have had a significant effect on BALF PIIINP or BALF ET-1 concentration.

One of our aims was to study the distribution of the histopathological lesions in WHWTs with CIPF. This information is important when lungs are sampled for histopathological examination in clinical practice. Unfortunately, we only had lobe specific lung samples from nine WHWTs with CIPF. We noticed that in dogs with mild diffuse fibrosis, the multifocal areas were more often located in the caudal than in the other lung lobes, but in dogs with moderate diffuse fibrosis, the fibrosis accentuation areas were equally seen in cranial and caudal lung lobes. The small number of lobe specific samples impeded us from making stronger conclusions.

For collagen and IHC staining, we selected only a single site of lung from each WHWT with CIPF. The site with the most severe lesions was chosen, which we expected to best correspond to lesions in human UIP. Optimally, several sites would have been evaluated. Therefore, it can be questioned whether choosing only one lung site for further staining influenced the comparison between CIPF and human UIP and NSIP. We investigated the maturity of fibrosis by collagen staining. By IHC staining we investigated qualitatively whether PCII hyperplasia, SM hyperplasia and bronchiolar metaplasia were present in affected canine lung, as these changes are characterized in human UIP. For general comparison between the canine and human diseases, the whole histologic picture from different HE stained lung sections, consisting of the pattern and degree of interstitial fibrosis, presence of honeycombing and inflammation, and distribution of lesions, was taken into account.

Only one of the three dogs we used as healthy controls in the histopathological study was of similar age to the diseased dogs. Based on this, we were not able to make any observations about possible age-related changes in the lung tissue.

6.5 FURTHER RESEARCH

For a long time CIPF has been considered a rare disease in dogs, but is this an outdated assumption? As veterinarians and dog owners become more familiar with the disease, it seems that CIPF is diagnosed more and more often, at least in Finland. It is worrying that in recent decades the incidence of human IPF has been rising (Navaratnam et al., 2011). In dogs, there are no estimates of the prevalence and incidence of CIPF. An epidemiological study would help us understand how widespread the problem we are dealing with is, how frequent CIPF is among WHWTs. Accurate answers are desired by the owners of the WHWTs and all enthusiasts of the breed. There is incentive for us to learn whether CIPF is an emerging disease. If this proves to be true, we should hurry to search for the reason.

Dogs with CIPF are commonly treated with corticosteroids despite there being no evidence to support their use. To date, no treatment trials have been performed on dogs with CIPF. In humans, corticosteroid therapy is no longer used to treat IPF, but it has a role in treating patients with NSIP (Kim et al., 2006; Raghu et al., 2011). Our study showed that dogs with CIPF have histopathological features of both of these human diseases. It would be important to know whether dogs truly benefit from corticosteroid treatment as do many people with NSIP, or whether the treatment does more harm than good. Investigating the potential effect of corticosteroid therapy on clinical signs and findings, arterial oxygenation,

HRCT features and on outcome might give us new insight into the pathophysiology of the disease. The interpretation of corticosteroid treatment trial results on CIPF, however, are likely complicated by corticosteroids potentially having an effect on the concomitant tracheal and bronchial problems of the CIPF dogs.

CIPF is a disease of WHWTs. Reports of the disease in other breeds of dogs are scarce. Lobetti et al. (2001) described the disease in Staffordshire bull terriers which were considerably younger than WHWTs usually are when diagnosed with CIPF. A question arises as to whether CIPF is the exact same disease across dog breeds. Could there be differences in CIPF of a WHWT and CIPF of a dog of another breed? Perhaps there is a variety of fibrotic ILDs affecting dogs, CIPF being only the tip of an iceberg. A combined clinical and histopathological study looking in detail at the patterns of fibrosis and clinical findings could answer these questions. As CIPF is seldom found in dogs other than WHWTs, a multicentre approach could be useful in obtaining adequate cases for study.

It is very challenging to try to diagnose CIPF at an early stage, before a dog shows any clinical signs. HRCT is probably one of the most sensitive means to detect subtle changes in the lung. New, faster CT scanners make this imaging method more feasible, as anaesthesia may not be needed for the procedure. HRCT scans might also be a way to monitor disease progression. We lack understanding about the histopathologic correspondence of the HRCT changes associated with CIPF, however. In humans, GGO, for example, might reflect different pathologic processes including inflammation or fibrosis beyond the resolution of the CT (Gotway et al., 2007). Which one is it in CIPF? Are the areas of GGO evolving to honeycombing and traction bronchiectasis? To make the most of the HRCT images, a study comparing the HRCT changes and the histopathologic appearance of those exact areas at the same time point would be of great value.

As our studies indicated, biomarkers can be useful aids in diagnosing CIPF. In addition to being part of the diagnostic puzzle, biomarkers could offer much more: they could be of prognostic value. To take the thought even further, the ideal biomarker would be able to predict which individual was going to get the disease and which was going to remain healthy. Such a biomarker does not exist, yet. Maybe a combination of various biomarkers would prove to be most helpful. There is much research needed in this field.

Matrix metalloproteinases (MMPs) are a group of proteases which participate in the remodelling of lung extracellular matrix (Oikonomidi et al., 2009), a process crucial to the development of CIPF. Our pilot study already showed increased MMP-2 and -9 activity in dogs with CIPF in comparison to CB (Heikkilä et al., 2011). In addition to these two MMPs, other MMPs are also implicated in the pathogenesis of human IPF. MMP-7 has shown potential as a biomarker predicting the progression of IPF and correlating with the severity of the disease in humans (Rosas et al., 2008; Song et al., 2013). The role of MMP-7 has not been studied in dogs with CIPF and it is therefore an obvious candidate for future research.

Many other aspects of CIPF require further clarification, such as the heritability of CIPF. It is likely to be a complex genetic dilemma but it must be resolved. Genetic studies can only be successful if the participating WHWTs with CIPF and control WHWTs are correctly diagnosed. A correct diagnosis is based on a well established phenotype, both clinically and histopathologically. Biomarkers may be of help. Our studies therefore provide a good basis on which further studies can build.

7 CONCLUSIONS

1. CIPF affects older WHWTs. The typical clinical sign is the combination of cough and exercise intolerance. Abdominal breathing pattern and Velcro crackles are often present. Thoracic radiographs show a bronchointerstitial lung pattern. CIPF does not induce specific haematological or serum biochemical alterations, and leads to significant hypoxemia. CIPF dogs have bronchoscopically detectable airway changes, and an increase in BALF TCC. On HRCT, GGO appears to be typical of CIPF but reticular changes are also noted.
2. Serum ET-1 concentration is elevated in dogs with CIPF in comparison to dogs with CB, EBP or healthy dogs. It can be used with good accuracy to differentiate between CIPF and CB. BALF ET-1 concentration is elevated in CIPF when compared to CB. CIPF is also associated with an elevated BALF PIIINP concentration, which can be used as a marker for differentiating CIPF from CB. Dogs with EBP also have high BALF PIIINP values. Serum PIIINP cannot be used to detect lung diseases in dogs.
3. CIPF is characterised by two types of interstitial fibrosis: a diffuse and mature fibrosis affecting the alveolar walls, and a multifocally accentuated, less mature fibrosis. Profound alveolar epithelial and luminal changes and occasional honeycombing accompany the areas of more severe fibrosis. Fibroblast foci are not a feature, but scattered myofibroblast are present in the interstitium. Areas of more severe fibrosis are predominantly located around the bronchioli or under the pleura. Based on these results, we conclude that CIPF shares features of both human UIP and NSIP.

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